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INTERACTION AMONG MAST CELLS, T-LYMPHOCYTES,
MACROPHAGES, AND ANTIGEN

Stewart G. Greisman

1981

YALE



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INTERACTION AMONG MAST CELLS, T-LYMPHOCYTES,
MACROPHAGES, AND ANTIGEN

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March, 1981

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ABSTRACT

Based upon previous experiments [4,22,55] a hypothesis was formulated that the local release of serotonin (5-hydroxytryptamine) from tissue mast cells was required to elicit delayed-type hypersensitivity in mice. There is substantial evidence that sensitized T-lymphocytes interact with mast cells leading to mast cell release of serotonin. This serotonin causes the separation of endothelial cell junctions, allowing the diapedesis of bone marrow derived effector cells into the delayed-type hypersensitivity (DTH) reaction site. The present study was undertaken to further characterize the T-cell - mast cell interaction in DTH and to obtain an in vitro correlate for this interaction.

There are at least four possible mechanisms by which T-cell - mast cell interaction, leading to serotonin release from mast cells, can occur: 1) via a T-cell derived lymphokine-like material, 2) via T-cell interaction, either direct or by means of a soluble factor, with macrophages which then activate mast cells, 3) via IgE, or 4) via a T-cell derived antigen specific molecule. Experiments with peritoneal exudate cells, a source of macrophages, revealed the presence of an inhibitor of background (spontaneous) serotonin release from mast cells along with some indirect evidence for the presence of a substance that could actually induce serotonin release. Other experiments identified an IgE-like substance on the surface of mast cells from mice immunized to optimally express DTH. Evidence was presented that this substance could be derived from T-cells.

INTRODUCTION

Immunology, as a separate discipline, is a relative newcomer to the modern scientific world. Immunology has emerged as a specialized field of study and research only since the middle of the twentieth century. The earliest historical references to immunology in the western world, however, date back to the 18th century. Reports of successful immunization against the disease of smallpox were made by Lady Mary Wortley Montague in 1721 and by Edward Jenner in 1789.

Immunology is now classically divided into two distinct, though often functionally complementary, systems: humoral (antibody) immunity and cell-mediated immunity. Less than one hundred years ago, the distinction between these two immunologic systems was not so evident. After Louis Pasteur's success with the attenuation of pathogens and with immunizations against anthrax and most notably rabies, two schools of thought emerged regarding immunity. One school was led by Elie Metchnikoff, the discoverer of phagocytosis, who in the 1880's espoused the theory that immunity was dependent on certain cells in the body. Support for the humoral theory of immunity came from the work of Emil von Behring who in the 1890's discovered that materials in the cell-free portion of blood, which were termed antitoxins, were capable of inactivating diphtheria and tetanus toxin. Eventually it became clear that both theories with regard to immunity were correct. Additional insight into cellular immunity came from the work of Robert Koch. Around the turn of the century, Koch described erythematous and indurated skin reactions which appeared some time after subcutaneous injection of tubercle bacilli

into guinea pigs already infected with the bacilli. Koch is thus credited with the discovery of delayed-type hypersensitivity reactions [9].

Delayed-type hypersensitivity

Delayed-type (Type IV) hypersensitivity reactions (DTH) are the prototype of cell-mediated immunity. As was described by Koch, DTH reactions are classically associated with immunization via infection with mycobacteria or with immunization augmented by water-in-oil emulsions containing mycobacteria (complete Freund's adjuvant). These reactions can be elicited by purified protein derivative of tuberculin (PPD), which is the antigen in mycobacteria most relevant to the expression of DTH responses, or by antigens that are administered with the mycobacteria. It has been well established that T (thymus derived)-lymphocytes play an integral role in DTH or cell-mediated reactions. B (Bursa of Fabricius equivalent)-lymphocytes, in contrast, have been identified as being primarily responsible for antibody-mediated or humoral immune reactions. T-lymphocytes have been shown to have other functions such as helping B-cells produce antibodies, regulating and suppressing immune responses and mediating cytotoxicity.

DTH reactions, elicited in guinea pigs previously infected with mycobacteria or immunized with complete Freund's adjuvant (CFA), are characterized macroscopically by erythema and induration, and microscopically by a predominance of mononuclear cells. There are, however, other types of delayed reactions which can be elicited in guinea pigs immunized with antigens administered in saline or

in non-mycobacteria containing water-in-oil emulsions (incomplete Freund's adjuvant). As compared to classic DTH or tuberculin reactions, these skin reactions are characterized by their lack of induration and their early disappearance after immunization and skin testing [10,11,42,50]. It was also noted that these delayed skin reactions were quite similar to reactions in humans called Jones-Mote reactions [50]. Jones-Mote reactions are delayed skin reactions produced in individuals previously immunized with protein antigens in saline [29,41].

Basophils and mast cells

An additional histologic feature of these delayed reactions induced by immunization without mycobacterial adjuvants is the prominent accumulation of basophils [18]. Based upon this observation, these delayed reactions have been termed cutaneous basophil hypersensitivity (CBH), as compared to the classic tuberculin-type reactions which essentially lack basophils [16]. Although being readily demonstrable in guinea pigs and in humans [64], CBH reactions have not been found to occur in mice. This finding has been attributed to the fact that mice essentially lack basophils [2]. Another interesting feature of this species has been the inability to elicit DTH reactions after flank skin testing in appropriately sensitized mice [2]. Good DTH reactions have, however, been obtained recently by testing in the ear skin (contact sensitivity [1]) or in the footpads [7]. Histologic examination of the normal skin of mice revealed greater numbers of vasoactive amine containing mast cells in the ear skin and in the footpads as compared to the flank skin [22]. This was

one of the first hints that mast cells seemed to play a crucial role in DTH reactions in mice.

Mast cells and basophils are similar in many respects. Both cells contain metachromatic granules in which vasoactive amines, such as histamine and serotonin (5-hydroxytryptamine), are stored. Both cells also possess F_c receptors on their surfaces which can bind IgE and certain subclasses of IgG via specialized domains in the F_c portion of these immunoglobulins. After this passive sensitization of the mast cell or basophil, if the proper multivalent antigen binds to the F_{ab} portion of the immunoglobulin, the antigen-antibody interaction then triggers the cell to degranulate and release its vasoactive amines. This process is known as immediate (Type I) hypersensitivity.

Mast cells are mononuclear cells that are normal inhabitants of various tissues, located preferentially around small blood vessels and near epithelial surfaces [45]. Mast cells probably originate in the bone marrow from a mesenchymal precursor. Mast cells, however, do not develop in the bone marrow and do not circulate in the blood stream. They circulate in some immature form, enter the tissues, and differentiate into granule-containing cells [30]. The local environment in the tissue is believed to play an instrumental role in mast cell differentiation. Basophils, as opposed to mast cells, are polymorphonuclear leukocytes which arise in the bone marrow from the same stem cell as the other polymorphonuclear leukocytes, eosinophils and neutrophils [17]. In contrast to mast cells, basophils are normally present in the bone marrow at different stages of development. A further distinction between basophils and mast cells is that basophils

are normally present in the blood, constituting approximately 0.5% of circulating white cells in humans. Mast cells, on the other hand, are solely located in the extravascular tissue spaces. In pathologic conditions, CBH reactions demonstrate that specific immune stimuli can, however, recruit basophils to enter tissues.

Role of mast cells in delayed-type hypersensitivity

DTH reactions are characterized by erythema, induration, and mononuclear cell infiltrates. Histologic evidence in mice has revealed substantial numbers of mast cells in normal skin of sites (ear skin and footpads) that preferentially express DTH [22]. Further studies of mast cells in DTH have shown that the release of vasoactive amines from mast cells is required for the elicitation of DTH. The role of vasoactive amines was examined in mice depleted of serotonin by injection of reserpine. Murine mast cells contain both serotonin and histamine but serotonin seems to be the principle amine because the vasculature of mice is insensitive to histamine [55]. Reserpine depletes mast cells of serotonin by competitive displacement of serotonin from its binding sites in the granules. The serotonin is thus rendered susceptible to intracellular degradation by monoamine oxidase (MAO). Mice treated with reserpine are unable to express DTH [22]. However, MAO inhibitors which block the catabolism of intracytoplasmic serotonin have been shown to restore DTH in mice treated with reserpine [22]. This observation indicated that reserpine treatment did not adversely affect lymphocyte

function or any other aspect of DTH. The lack of serotonin was hence most likely responsible for the inability to elicit DTH.

Other studies have also shown that the local release of serotonin (5-HT) is required to elicit DTH in mice. Anti-histamines were found to have no effect of DTH reactions, but cyproheptadine, a serotonin and histamine antagonist, inhibited these reactions [2]. Autoradiographic experiments have revealed that tritiated serotonin (^3H -5HT) was localized to tissue mast cells after systemic injection [22]. Further experiments showed that ^3H -5HT was depleted from mast cells in DTH, accompanied by the formation of gaps between endothelial cell junctions [4]. This observation was consistent with a previously described action of 5-HT to increase vascular permeability allowing the extravasation of various tracers into the extravascular space [36].

Role of T-lymphocytes in delayed-type hypersensitivity

Numerous lines of evidence have thus established the crucial role of mast cells in DTH; mast cell activation provides the necessary serotonin for the amine dependent diapedesis of bone marrow derived cells in DTH reactions. It is also well established that sensitized T-lymphocytes play a significant role in DTH. Upon antigenic challenge, T-cells release soluble products called lymphokines, some having the ability to recruit effector cells. Additional experiments have examined the role played by T-cells in DTH, particularly in regard to mast cell activation. A murine model of DTH was utilized for these studies.

Mice received an optimal immunization for the elicitation of DTH (0.2ml of 0.01% sheep erythrocytes [SRBC]). Four days later, the mice were tested for DTH by injecting SRBC into a footpad. The DTH response was quantitated by measuring footpad thickness before and after antigenic challenge [7].

The sheep erythrocyte DTH system has many similarities to cutaneous basophil hypersensitivity and Jones-Mote reactions. These three examples of DTH are delayed reactions, but in contrast to classic tuberculin reactions that are achieved by immunization with mycobacteria, they tend to be elicitable for only a short period of time after immunization. Using the SRBC model, DTH in mice was maximally elicitable four days after SRBC immunization but by 25 days, there was no detectable DTH reaction. It was postulated that this transient phenomenon was a result of effector T-cells with an intrinsic short life span [7].

The SRBC murine model of DTH has also been utilized to study the ability of immune cells to transfer DTH to non-immune recipients. In such experiments, donor mice were optimally immunized to express DTH with 0.01% SRBC. At day four, their spleen cells were harvested and transferred intravenously to syngeneic non-immune recipients. The recipients were then challenged with footpad injections of SRBC and were found to have significant DTH reactions [7]. Using autoradiography and electron microscopy, it has been demonstrated mast cells in recipients of immune spleen cells undergo activation with 5-HT release and endothelial cell changes, similar to those seen in actively sensitized animals [4].

Other experiments involving the transfer of immune cells also support mast cell activation by T-cells in DTH. As mentioned previously, mice treated with reserpine are unable to express DTH. Immune spleen cells were unable to transfer DTH to non-immune recipients which were pretreated with reserpine. Reserpine treated mice, themselves unable to express DTH when skin tested, were, however, able to serve as cell donors for the transfer of DTH to non-immune recipients [22].

Further studies using these transfer techniques have uncovered additional evidence supporting the role of T-cells in DTH. Immune spleen cells were fractionated on a nylon wool column before transfer to non-immune mice. The fraction of non-adherent, non-immunoglobulin bearing lymphocytes from the column was presumed to contain only T-cells, being essentially void of B-cells. This fraction was able to transfer DTH responses to recipients, along with the capacity for mast cell activation, as determined by the release of ^3H -5HT, and for endothelial gap formation [4].

Evidence against the involvement of B-lymphocytes in delayed-type hypersensitivity

The SRBC model of DTH also provides evidence against the participation of B-cells. Four days after 0.01% SRBC immunization, when DTH was found to be optimally elicitable, B-cells were found to have not been stimulated by the SRBC immunization. This lack of stimulation was determined by noting a lack of antibodies against SRBC [6] and a lack of induction of antigen-binding, rosette-forming B-cells [14]. An immunization dose of

0.01% SRBC was found to selectively induce rosette-forming T-cells, while rosette-forming B-cells were actually suppressed below normal background levels [14]. Higher immunization doses, 10% SRBC, however, selectively induced rosette-forming B-cells while suppressing rosette-forming T-cells [14]. These higher immunization doses also induce antibody formation. The functional role of these rosette-forming cells in the immune response is unknown.

Further evidence against the involvement of B-cells in DTH comes from mice treated with anti-IgM antisera. These mice were rendered agammaglobulinemic and were unable to make antibody responses. These mice have, however, intact DTH responses [12,35]. Additional studies used mice immunized with high doses of SRBC (10%) and pretreated with B-cell depleting doses of cyclophosphamide. Mice receiving only high doses of SRBC have poor DTH responses; cyclophosphamide pretreatment in these animals augmented DTH responses by eliminating suppressor activity [7,22]. Mice receiving high doses of SRBC and cyclophosphamide have essentially no detectable antibody or B-cells. DTH responses of these mice did show mast cell activation with the formation of gaps between endothelial cells [4]. Thus the ultrastructural changes following mast cell activation in DTH were T-cell, not B-cell, dependent.

Present study: In vitro interaction among mast cells, T-lymphocytes, macrophages, and antigen

As the preceding studies have shown, considerable evidence has been accumulated in support of the hypothesis that after antigenic challenge, specifically sensitized T-cells

interact in some way with local mast cells. Mast cells then release serotonin which, in turn, causes the formation of gaps between endothelial cell junction, permitting the diapedesis of bone marrow derived cells to the site of antigenic challenge. This site is recognized macroscopically as a DTH reaction. It is interesting to note that T-cells are able to pass directly through the cytoplasm of endothelial cells, a process known as emperipolesis. Other circulating cells are only able to pass through gaps between endothelial cells to reach extravascular spaces [38]. Another feature of T-cells is their ability, upon antigenic challenge, to secrete lymphokines, some of which are chemotactic for bone marrow derived leukocytes. As the experiments with serotonin antagonists have demonstrated, these chemotactic lymphokines along with the other lymphokines which increase vascular permeability [28] are not sufficient to produce the cellular infiltrates characteristic of DTH reactions. It thus appears crucial for T-cells to collaborate in some manner with mast cells for the elicitation of a DTH reaction.

The present study focuses on this T-cell - mast cell collaboration. Based upon the information presented pertaining to the in vivo aspects of the T-cell - mast cell interaction, various experiments were performed attempting to identify this interaction in vitro. There are at least four possible mechanisms through which this collaboration, leading to mast cell activation in DTH, can occur: 1) via a T-cell derived lymphokine-like material, 2) via T-cell interaction, either direct or by means of a soluble factor, with macrophages which then activate

mast cells, 3) via IgE, or 4) via a T-cell derived antigen specific molecule, "IgT," which possesses IgE-like activity.

Hypothesis 1: A T-cell derived lymphokine activates mast cells in DTH

The hypothesis that a T-cell derived product mediates mast cell activation is compatible with recent studies. A multitude of lymphokines have been described with a wide range of biologic activities [49]. A lymphocyte derived factor which possesses chemotactic properties for basophils has been identified [32,63]. As previously noted, basophils are in many respects quite similar to mast cells, both being involved in IgE dependent reactions [33] and in complement mediated reactions [24]. Both cells also play major roles in DTH (basophils in cutaneous basophil hypersensitivity) reactions. It has recently been demonstrated that supernatants generated from human mononuclear cell cultures were able to induce histamine release from basophils in vitro. Production of this activity was stimulated by mitogens and by antigens that cause DTH skin reactions. These experiments have provided evidence for a collaboration between lymphocytes and basophils via a soluble mediator [58]. In a somewhat different system, it has been shown that the immunologic stimulation of human leukocytes in culture resulted in the release of soluble mediators that were able to augment IgE mediated histamine release [27].

To test for the presence of a lymphokine promoting mast cell activation, various supernatants were generated from the incubation of immune cells with antigens that are known to cause DTH reactions (PPD, SRBC). These supernatants were then incubated

in vitro with mast cells. The assay for mast cell activation is described later.

Hypothesis 2: Macrophages serve as an intermediary for T-cell activation of mast cells

Another hypothesis for T-cell - mast cell interaction is that macrophages act as an intermediary between T-cells and mast cells. Macrophages play important roles in a variety of immunologic reactions [60,61]. Macrophages are required for T-cell production of lymphokines in response to antigenic stimulation. Before T-cells can recognize and respond to antigen, macrophages must first take up and process the antigen [62]. Physical association between T-cells and antigen-presenting macrophages is also required for T-cell proliferation [52]. This contact is probably mediated at some portion of the major histocompatibility complex because macrophages and T-cells must be syngeneic at the Ia locus of the H-2 MHC for optimal interaction to occur [53]. Since DTH responses are classically evoked by antigenic challenge in skin, much research has focused on identifying a macrophage-like cell in the skin. Cells residing within mammalian epidermis, Langerhans cells, have recently been shown to be related functionally to monocytes and macrophages [57].

To identify macrophage participation in the interaction between mast cells and T-cells, these three cells were incubated together and the assay for mast cell activation was performed. Peritoneal exudate cells, induced by the intraperitoneal injection of complete or incomplete Freund's adjuvant, served as antigen-presenting cells. These cells have been shown to be proficient

in antigen presentation and in their ability to interact with lymphocytes [5]. Spleen cells from immunized mice served as the source of sensitized T-cells.

Hypothesis 3: IgE mediates T-cell activation of mast cells

Another proposed hypothesis is that IgE is the mediator for T-cell - mast cell interaction in DTH. IgE is known to bind to mast cells and immediate anaphylactic hypersensitivity is the result of mast cell degranulation caused by antigen combining with IgE. It has been demonstrated that after immediate-type hypersensitivity reactions, there is a late cutaneous phase with a delayed time course [3]. Various studies using immune serum transfers and testing with anti-IgE antibody have revealed that IgE mediated release of various vasoactive substances from cutaneous mast cells was involved in these late phase reactions [3]. Thus IgE can play a role in skin reactions with a delayed time course. Another antibody, IgG₁, has been observed to be able to mediate cutaneous basophil hypersensitivity [3]. Thus B-cells and antibodies, in addition to T-cells, seem to also play a role in certain delayed reactions. The clinical implications and differences between antibody mediated and cell mediated delayed reactions are not fully appreciated.

There is, however, a good amount of evidence against the participation of B-cells in the DTH model involving SRBC immunization and skin testing. As was presented earlier, DTH reactions were passively transferred with an enriched T-cell population after passage of immune spleen cells through a nylon wool column. Moreover, mice treated with B-cell depleting doses of cyclophosphamide

and immunized with large doses of SRBC were indeed able to express DTH. Furthermore, mice which were treated with anti-IgM antisera and rendered unable to mount an antibody response were also able to express DTH [12,35]. It is of course difficult to prove that all B-cells were eliminated by these techniques, and a few surviving B-cells may produce enough IgE to passively sensitize tissue mast cells.

To detect antibody on the surface of mast cells, peritoneal cells containing 3% mast cells were collected from immunized mice and incubated with a multivalent antigen. Mice were immunized with 0.01% SRBC, an optimal dose for the elicitation of DTH [7], and then mast cells were isolated and incubated with SRBC, assaying for mast cell activation. In control experiments, mice were immunized with antibody inducing doses of SRBC or saline. In other experiments, these different kinds of mast cells were incubated with anti-mouse IgE antibody and anti-mouse IgG antibody, attempting to detect, respectively, IgE and IgG on mast cell surfaces.

Hypothesis 4: A T-cell derived antigen specific molecule mediates T-cell activation of mast cells

The other proposed hypothesis is that T-cells interact with mast cells via a T-cell derived antigen specific molecule. T-cell recognition and interaction with antigens has for a long time been a subject of controversy. Immunoglobulin-like molecules have been isolated from T-cell membranes which appear distinct from B-cell membrane immunoglobulins on structural, functional, and serologic grounds [43]. Molecules on the surfaces of T-cells

have been found to possess idiotypic and immunoglobulin variable region framework determinants. These T-cell "receptors" have been discovered to also have antigen binding properties [20]. If this T-cell derived material had IgE-like properties also, it may be able to interact with mast cells.

If a T-cell derived substance possessed IgE-like activity and was able to attach to mast cells, it may be detectable by incubating mast cells with SRBC (antigen) or possible with anti-mouse IgE antibody. An assay for mast cell activation could then be performed. Antigen and anti-IgE antibody were incubated with mast cells from mice immunized with an optimal dose for DTH elicitation or with an antibody inducing dose in the hope that the antigen or the anti-IgE antibody might detect more IgE-like material on mast cells after the DTH immunizing dose.

Assay for mast cell activation

The activation of mast cells by various agents has been studied in vitro by the release of tritiated serotonin (^3H -5HT) [13,37,44]. Mast cells constitute approximately 3% - 5% of the peritoneal cells in mice. Mast cells in the mixed peritoneal cell population have been shown to actively take up and concentrate exogenous serotonin [44]. The ability of rat mast cells to incorporate and release ^3H -5HT has recently been used as an assay for mediator release. The parallel release of ^3H -5HT and endogenous histamine was observed after the incubation of mast cells with compound 48/80 [40], a non-specific mast cell secretagogue, or after the incubation of actively sensitized mast cells with antigen [56]. Other studies have shown release

of ^3H -serotonin from mouse mast cells by 48/80, Concanavalin A, and antigen and anti-mouse immunoglobulin using passively sensitized mast cells [37]. Regardless of whether 48/80 was incubated with whole peritoneal cell suspensions (3% - 5% mast cells) or 95% purified mast cell suspensions, ^3H -serotonin release was virtually identical [37]. This observation confirmed that only mast cells were being labelled by the tritiated serotonin.

In these various studies, peritoneal mast cells, representing 3% - 5% of the total cell population, were incubated with ^3H -5HT at 37°C . for varying amounts of time from 30 minutes [37] to three hours [13]. A 90 minute incubation seemed optimal for mast cell incorporation of ^3H -5HT. The cells were then washed to eliminate unincorporated ^3H -5HT. Washing the cells one to four times before incubation with mast cell stimulators progressively lowered non-specific background release [13]. Three washes were generally used to optimally reduce background release of ^3H -5HT. The various stimulators of mast cell activation and mediator release were then incubated with the ^3H -5HT labelled mast cells. After the incubation period with the stimulators, the mast cells were centrifuged and percent ^3H -5HT release was calculated by dividing the total number of counts in the supernatant by the total number of counts in the supernatant and pellet.

MATERIALS AND METHODS

Animals: Five-week old BDF₁ male mice were obtained from Jackson Laboratories, Bar Harbor, Maine and were rested a minimum of 1 week. All mice used in experiments were between 6 and 10 weeks of age, weighing 25-30 grams.

Induction of peritoneal exudate cells: 0.5ml of complete Freund's adjuvant (H37-Ra) or incomplete Freund's adjuvant (DIFCO Laboratories, Detroit, Michigan) was injected into the peritoneal cavity of mice. The CFA or IFA-induced peritoneal exudate cells (PEC) were harvested 3 to 24 days after injection. CFA-SRBC-PEC were induced by injecting 1.5×10^8 SRBC IP one hour before sacrificing a mouse which had, 5 days earlier, received 0.5ml of CFA IP.

SRBC immunization: Fresh sheep blood (Colorado Serum Company Laboratories, Denver, Colorado), stored in modified Alsevers solution and used within 2 weeks of delivery, was washed 3 times in sterile pyrogen free saline (Cutter Laboratories, Berkeley, CA) before injection. Mice were immunized by intravenous injection, via the retro-orbital plexus approached via the medial aspect of the eye, after being lightly anesthetized with ether. Mice were injected with 0.2ml of a 0.01% suspension of SRBC in saline (an optimal dose for DTH) or 0.2ml of a 10% SRBC suspension (an antibody inducing dose). These immunizations were used to generate both immune mast cells and immune spleen cells. Control animals were injected with 0.2ml of saline.

Isolation of mouse peritoneal mast cells: Peritoneal mast cells were collected from normal BDF₁ mice, or immune mice that had previously

received 0.01% SRBC or 10% SRBC IV, 4 or 18 days before sacrificing. The animals were sacrificed by cervical dislocation without anesthesia and 3 - 4ml of Locke's solution (NaCl-150mM, KCl-5mM, CaCl_2 -1mM, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-5mM, glucose-5.5mM, BSA-0.1%, osmolality-305mosm, titrated to pH 7.0 with NaOH) were injected into the peritoneal cavity. The abdomen was gently massaged for 60 seconds, and then the abdominal musculature was opened down the midline. The peritoneal cavity was opened and approximately 3.5ml of clear peritoneal fluid was withdrawn with a plastic pasteur pipette and introduced into a 50ml plastic Corning centrifuge tube. From this point on, all manipulations were performed at 4°C., including all centrifugations. The peritoneal cells were spun at 350 x g for 10 minutes and washed twice in Locke's solution. Unless specified, all subsequent centrifugations were done at 350 x g for 10 minutes (1200rpm in a Damon/IEC centrifuge, PR-6000, head radius of 23 centimeters). Platelets, as confirmed by microscopic examination of the peritoneal cells, were eliminated after the first spin. This step was important because only mast cells and platelets are known to incorporate exogenous serotonin. After these centrifugations, mast cells, lymphocytes, and other mononuclear cells were left in the pellet. For experiments such as those in Tables 4 and 5, where there was a possibility of some factor coating mast cells, only one wash was performed. The peritoneal cells were reconstituted in Locke's solution, and then counted in a hemocytometer with toluidine blue staining. Cell viabilities were determined by trypan blue exclusion.

^3H -serotonin labelling of mast cells: Mast cells, $2 \times 10^5/\text{ml}$, were then incubated with ^3H -5 hydroxytryptamine binoxalate (1.0mCurie/

0.0082mg/1.0ml, New England Nuclear, Boston, MA) at a concentration of 5uCurie/ 10^6 mast cells in a 37°C. water bath for 90 minutes.

After the incubation, the mast cells were washed three times to eliminate unincorporated ^3H -serotonin (5-HT). Only one wash was performed in Tables 4 and 5 because of the possibility of a factor coating the mast cells. The cells were then reconstituted so that 0.5ml contained 10^5 mast cells which was then added to each assay tube (5ml polystyrene Falcon tubes, #2058, Oxnard, CA). In the various experiments, different supernatants, RBC, antibody, etc. were added to the mast cells and the total incubation volume was adjusted to 1ml with Locke's solution. The mast cells were, unless specified, incubated for 90 minutes in a 37°C. water bath.

48/80 (Sigma Chemicals), a non-specific mast cell secretagogue, and media controls were included in all experiments.

Determination of ^3H -serotonin release: After the various mast cell incubations, the tubes were spun at 350 x g for 10 minutes. When mast cells were incubated with 1% SRBC or 1% HRBC, the assay tubes were spun at 450 x g (1500rpm, head radius of 23cm) for 10 minutes because of the increased size of the pellet. Each tube's supernatant, which had a volume of approximately 1ml, was poured off and divided evenly between two 5ml glass scintillation vials. The pellet was resuspended in 1ml of a 1% solution of Triton-X (New England Nuclear), vortexed, and also divided evenly between two scintillation vials. Thus each vial had approximately 0.5ml of supernatant or resuspended pellet. 3.5ml of Aquasol (universal liquid scintillation counting cocktail, New England Nuclear) was added to each vial. Each vial was then vortexed to obtain a homogeneous solution of the aqueous solution and Aquasol. The vials were then counted in

a Beckman β -counter, (Model LS-330) for 1 minute. To calculate percent ^3H -serotonin release, the average number of counts/minute in the two supernatant vials, which represents ^3H -5HT released from mast cells, was divided by the total number of counts, determined by adding the average counts/minute in the two supernatant vials and the average counts/minute in the two pellet vials. This method used to calculate the percent ^3H -5HT release does not require having the same number of mast cells in each assay tube. In a typical experiment, the average number of counts in the supernatant was in the thousands and the average number of counts in the pellet was in the tens of thousands. Vials containing scintillation fluid with Locke's solution had counts of less than 50.

Incubation of ovalbumin-induced supernatants and mast cells:

Various supernatants were obtained for incubation with ^3H -5HT labelled mast cells, attempting to identify a lymphokine-like material that caused 5HT release from mast cells. Ovalbumin-induced supernatants were generously supplied by Dr. Nancy Ruddle, Laboratory of Environmental and Public Health, Yale University, New Haven, CT. These supernatants were prepared by incubating inguinal lymph node cells with 250ug/ml of ovalbumin (OVA). The lymph node cells were harvested 8 days after subcutaneous injection of 50ug OVA + CFA into the tail of C57 B16 female mice. Supernatants were generated from a 24 hour incubation in a plate or conical tube of LNC, 2×10^7 /ml, and antigen. These supernatants were originally generated for lymphotoxin measurement. The percent killing of A-9 fibroblasts for a 1:2 dilution of supernatants from the plate and conical tube incubations was respectively, 69% and 39%. To assay for ^3H -5HT release (Table 1), 500ul, 50ul, and 5ul of the two supernatants and media were added to 0.5ml of

^3H -5HT labelled mast cells and the volume in the various assay tubes was adjusted to 1ml with Locke's solution. Thus the different supernatant and media dilutions were 1:2, 1:20, and 1:200. The final mast cell concentration was 10^5 cells/ml. The tubes were incubated for 90 minutes at 37°C . The tubes were then processed as described above under determination of ^3H -5HT release.

Incubation of T-cell lymphoma and hybridoma supernatants and mast cells: These supernatants were also generously supplied by Dr. Ruddle. These supernatants were generated from T-cells from a lymphoma parent strain, BW5147, and from a hybridoma strain, 64C11. These cells were previously sensitized to ovalbumin but were incubated for 48 hours in the absence of ovalbumin. The T-cells were incubated both with and without fetal calf serum in the media because different lymphokines are produced under the different media conditions. The supernatants generated after 48 hours are known to contain various lymphokines, including lymphotoxin and interferon. For the ^3H -5HT release from mast cell assay (Table 2), the supernatants and media controls were diluted in Locke's solution, incubated with mast cells for 90 minutes at 37°C ., and processed as described earlier.

Incubation of PEC and mast cells: CFA and IFA-PEC were induced as described earlier. The cells were harvested three days after IP injection (experiments shown in Figure 5) or five or nine days after injection (experiments shown in Figure 6) using sterile technique. The PEC were harvested in the same manner as normal peritoneal cells except that the cells were isolated in Hanks' balanced salt solution (Grand Island Biological Company, Grand

Island, NY) + 10% FCS. The PEC were washed three times, counted, and brought up to 7×10^6 cells/ml in RPMI 1640 (GIBCO) supplemented with 5% FCS (Hy-Clone, heat inactivated, Sterile Systems, Inc., Logan, Utah), 100 IU/ml penicillin, 100 ug/ml streptomycin, 75 ug/ml gentamicin and 1% freshly thawed L-glutamine. Various concentrations of PPD (Connaught Medical Research Laboratories, Willowdale, Ontario), 667, 250, 50 and 5 ug/ml, were added and the PEC + PPD were incubated in flat-bottomed circular wells with a diameter of 3 cm (Costar, Cambridge, MA) in a 37°C . CO_2 incubator for 24 hours (Figure 5) or 48 hours (Figure 6). After the incubation, the PEC cultures were spun and aliquots of the different supernatants were taken and diluted along with the media in Locke's solution. The supernatant and media controls were then incubated with ^3H -5HT labelled mast cells for 90 minutes at 37°C ., and processed as described above. In Figures 5 and 6, 0.5ml of the different supernatants and media were added to 0.5ml of mast cells, hence a 1:2 supernatant dilution. The experiment shown in Figure 7 was performed similarly to those in Figures 5 and 6, except that the PEC \pm PPD incubations were carried out in 15ml Corning conical centrifuge tubes at a concentration of $15 - 20 \times 10^6$ PEC/ml or 6×10^7 cells/tube.

Incubation of PEC and T-cell derived supernatants and subsequent incubation of the supernatant with mast cells:

T-cell derived supernatants were generously supplied by Dr. Charles Janeway, Immunology Section, Department of Pathology, Yale University. These supernatants were generated from T-cell clones and hybridomas under various stimulatory conditions. T-cell supernatants were also supplied by Drs. Monte Meltzer and Edward J. Leonard, Biology Branch,

Cancer Center, National Institutes of Health. These supernatants were generated from the incubation of immune cells and antigen and are known to contain various lymphokines. In addition to incubating 0.5ml of these supernatants directly with mast cells, these supernatants were also incubated with three day CFA and IFA-PEC in wells for 24 hours at 37°C. (Table 3). After this incubation, these supernatants were then incubated with ^3H -5HT labelled mast cells in 5ml Falcon tubes, and processed as described earlier.

Incubation of mast cells, T-cells, antigen and macrophages:

Spleen cells were used as the source of T-cells. Spleens from mice immunized 4 days earlier with either 0.2ml of 0.01% SRBC or 0.2ml of 10% SRBC or saline were isolated, using sterile technique. Spleens from a minimum of two animals were combined as a source for each type of immune or normal spleen cell. The spleens were squeezed into a cell suspension between two glass slides, washed twice in HBSS + 10% FCS and brought up to 7×10^6 cells/ml in RPMI 1640 + 5% FCS, penicillin, streptomycin, gentamicin, and fresh L-glutamine. CFA-SRBC-PEC, induced and isolated as previously described, served as the source of antigen (free SRBC) and antigen-presenting cells or macrophages. 10^5 ^3H -5HT mast cells were added to 1ml of each of the three kinds of spleen cells (0.01% SRBC, 10% SRBC, and normal) and 1ml of CFA-SRBC-PEC. The different combinations of these cells (Figure 8a) were centrifuged 350 x g for 10 minutes attempting to optimize cell-cell interaction, and incubated in the assay tubes for 90 minutes at 37°C. In a similar experiment (Figure 8c), the same protocol was followed except that the mast cells, CFA-SRBC-PEC and spleen cells were allowed to

settle in the tubes during a 24 hour incubation period. In another experiment (Figure 8c), CFA-SRBC-PEC + spleen cells were incubated for 24 hours in tubes. The supernatant and non-adherent cells were then decanted off and 10^5 ^3H -5HT labelled mast cells were spun down onto the adhered cells and incubated for 90 minutes at 37°C .

Interaction between RBC and mast cells: Mast cells from normal and immune mice (4 or 18 days after 0.01% SRBC or 4 days after 10% SRBC) were isolated and labelled with ^3H -5HT. 0.5ml of a 1% solution of SRBC or HRBC in Locke's solution (5×10^7 RBC/ml) was added to 10^5 mast cells in 0.5ml (100 fold excess of RBC). The RBC + mast cells were incubated under the following conditions: 1) pelleted, then 45 minutes at 4°C ., 45 minutes at 37°C ., 2) settling, 45 minutes at 4°C ., 45 minutes at 37°C ., 3) settling, 90 minutes at 37°C . These experiments (Table 4) attempted to identify an IgE-like material on the surface of mast cells from immunized mice. The first two incubation conditions were performed in an effort to simulate rosetting conditions. The mast cell, theoretically, would first interact with the RBC at 4°C ., and then, this interaction would trigger the mast cell to release ^3H -5HT at 37°C ., a process known to require metabolic energy.

Interaction between mast cells and anti-immunoglobulin antibody:

Anti-DNP IgE was generously supplied by Dr. David H. Katz, Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, La Jolla, CA. The anti-DNP IgE was obtained from a murine hybridoma which secretes monoclonal IgE antibodies of anti-2,4-dinitrophenyl (DNP) specificity [34]. Rabbit anti-mouse

IgE, also supplied by Dr. Katz, was produced by immunizing a rabbit with the anti-DNP IgE hybridoma. Goat anti-mouse IgG was generously supplied by Dr. Robert Cone, Immunology Section, Department of Pathology, Yale University. This antibody was obtained by the affinity purification of antibody from goats immunized with mouse IgG. The final protein concentration was 0.35mg/ml. The different antibodies were incubated with the previously described four different kinds of mast cells (Table 5). In one set of assay tubes, anti-DNP IgE (1:40 dilution of the stock) was incubated with the different mast cells for 45 minutes at 37°C. The tubes were then spun and the supernatant was poured off and saved to be counted later. The pellets were brought up in 1ml of Locke's solution and the rabbit anti-mouse IgE (1:40 dilution of the stock) was added and incubated for an additional 45 minutes at 37°C. Thus the anti-mouse IgE, in this case, was acting in a manner similar to antigen by bridging the anti-DNP IgE on the mast cell surface, triggering ³H-5HT release.

Rabbit anti-mouse IgE and goat anti-mouse IgG were also added directly to the four kinds of mast cells to detect whether there was any immunoglobulin-like material already present on the mast cell surface. This material would be cross-linked by the antibody, thereby triggering mast cell degranulation. The mast cells were first incubated without any additive in Locke's solution for 45 minutes at 37°C. (corresponding to the anti-DNP IgE incubation) and then the rabbit anti-mouse IgE (1:40 dilution) or the goat anti-mouse IgG (1:40 dilution) were added and incubated for an additional 45 minutes. The tubes were then processed as described

earlier.

Data analysis: All statistical comparisons were performed using the two-tailed Student's t-test.

RESULTS

Profile of cell populations:

(1) Normal peritoneal lavage cells - Mast cells constitute only a small fraction of the total cell population in the peritoneal lavage from a normal mouse. Using a lavage volume of 4ml of Locke's solution, approximately 3×10^6 cells are obtained per mouse: 30% lymphocytes, 67% other mononuclear cells, and 3% or 1×10^5 mast cells. No polymorphonuclear leukocytes are observed in the lavage. Mast cells are detected by Wright-Giemsa or Toluidine blue stains (Figure 2). Viabilities, as measured by trypan blue exclusion, were greater than 97%. These counts did not vary significantly between normal or previously immunized mice.

(2) Complete or incomplete Freund's adjuvant induced peritoneal exudate cells - Peritoneal exudate cells (PEC), induced by 0.5ml of IFA or CFA, were collected from three to 24 days after intra-peritoneal injection. $30 - 45 \times 10^6$ PEC were obtained per mouse with viabilities of greater than 92%. The viability of the PEC obtained 24 days after injection was slightly less, approximately 85%. The differential cell count of the PEC was: 30% mononuclear cells with vacuolated cytoplasm (henceforth termed macrophages), 30% lymphocytes, and 40% neutrophils (Figure 2). PEC collected on different days after induction had similar counts except for a 5 - 10% increase in lymphocytes seen 24 days after induction. There were no identifiable mast cells.

CFA-SRBC-PEC were induced by injecting 1.5×10^8 SRBC IP one hour before sacrificing a mouse which had, five days earlier, received 0.5ml CFA IP. Previous studies with ^{51}Cr -labelled SRBC showed phagocytosis of SRBC by the PEC. CFA-SRBC-PEC were similar

to CFA-PEC microscopically, except for the presence of free RBC.

(3) Spleen cell population - $80 - 100 \times 10^6$ spleen cells were obtained per mouse with viabilities of 80 - 90%. There were no morphologic differences between spleen cells from immune or non-immune animals.

Background ^3H -serotonin release in Locke's solution: The mean \pm standard deviation background or spontaneous release of ^3H -serotonin (^3H -5HT) from mast cells ($10^5/\text{ml}$ in stationary round bottom 5ml polystyrene tubes) was $3.5\% \pm 1.4$ using an incubation period of 90 minutes at 37°C . Figure 3 shows that the background release increased as the serotonin preparation aged, reaching 16% after five months of storage. For this reason, each serotonin batch was only used for two months to keep background release at a minimum.

Background release of ^3H -5HT from mast cells was significantly influenced by the media used for incubation. Incubation in RPMI 1640 with 5% fetal calf serum caused a 3 - 5% increase in background spontaneous release above that obtained in Locke's solution. RPMI 1640 with 10% FCS caused a further increase of 2 - 3%. Thus it was always important to control for the particular media being used. It was noted that vigorous pipetting of labelled mast cells also tended to increase background release. Thus mouse mast cells were quite sensitive to manipulation.

Positive controls: Compound 48/80, a non-specific mast cell secretagogue, was used as a positive control. Figure 4 shows that higher concentrations of 48/80 caused greater ^3H -5HT release. The actual percentage of serotonin release at the different [48/80]

was similar to the release of serotonin from rat mast cells [37]. Total recoverable ^3H -5HT, as determined by exposing labelled mast cells to a 60°C . water bath for 15 minutes, was 96%.

Experiments attempting to identify a lymphokine-like material that caused serotonin release by mast cells: Based on the hypothesis that mast cells are activated in DTH via a T-lymphocyte derived lymphokine-like substance, various supernatants were generated in vitro from the interaction between immune cells and antigen. These supernatants were then exposed to ^3H -5HT labelled mast cells.

In Table 1, two types of ovalbumin induced supernatants, one generated from incubation between lymph node cells and antigen in a plate, the other from incubation in conical tubes, were incubated with mast cells. These supernatants are known to contain lymphotoxin activity. Because of the possible presence of an inhibitor, different supernatant dilutions were tested in an effort to unmask an agonist effect. Results show essentially no difference between the media controls and the supernatants. The release caused by a 1:2 dilution of the supernatant from LNC + OVA was not significantly greater than the media control. The various dilutions were made in Locke's solution. Since the media used here (MEM + FCS) caused higher background 5-HT release than Locke's, this probably explains the decrease in release at the higher dilutions.

Table 2 shows serotonin release after mast cells were incubated with supernatants from various T-cell lymphomas and hybridomas, incubated without antigen. These supernatants are known to contain lymphotoxin and interferon activity. Media with and without serum were used because different lymphokines are produced by the hybridomas

under the different media conditions. The results again show no significant release above background levels.

The next set of experiments involved incubating CFA or IFA induced PEC with PPD, which is the antigen in mycobacteria relevant to the elicitation of DTH responses. The supernatants generated from these PEC cultures were then incubated with ^3H -5HT labelled mast cells. As shown in Figure 5a, mast cell serotonin release after incubation with these supernatants was actually less than the background media release. The 5-HT release for the media without PPD and for media with the different PPD concentrations was $8.3\% \pm 0.5$ (mean \pm S.D.). The mean 5-HT release after mast cell incubation with supernatants from IFA or CFA-PEC was $4.7\% \pm 0.8$. These results are significantly different ($p < 0.01$, t-test). Figure 5b shows that this inhibition of spontaneous or background release was present at all [PPD] and in supernatants from PEC incubated without PPD. The inhibition also was independent of the type of PEC, whether induced by CFA or IFA. The mean inhibition for the two kinds of PEC at the different [PPD] was $43\% \pm 9.6$.

In the experiment summarized in Figure 6a, five day and nine day CFA-PEC were incubated for 48 hours with PPD, twice as long an incubation period as the experiments in Figure 5. Again, there was a significant inhibition of background 5-HT release after mast cell incubation with the various PEC supernatants ($p < 0.01$, t-test). Figure 6b shows that the mean inhibition of background release was $44\% \pm 6.3$, essentially identical to the inhibition of $43\% \pm 9.6$ with 24 hour supernatants (Figure 5b). Thus based upon Figures 5 and 6, the inhibition is independent of antigen, independent of the type of PEC, and is apparent after culture periods of 24 or

48 hours.

In the experiments depicted in Figures 5 and 6, the incubations between PEC and the antigen, PPD, were carried out in flat-bottomed circular wells with a diameter of 3cm at a cell concentration of 7×10^6 cells/ml. Recent reports [39,47,48] have demonstrated production of substantially higher titers of lymphokines (macrophage migration inhibitory factor) by incubating antigen with higher concentrations of immune cells and allowing them to settle in conical instead of round bottomed tubes. Using this information, $15 - 20 \times 10^6$ PEC/ml (6×10^7 cells/tube) were incubated with and without antigen in 15ml conical tubes for 48 hours (Figure 7) in an attempt to unmask the effects of a possible inhibitor by potentially increasing lymphokine production. Various dilutions of these supernatants were incubated with mast cells, again attempting to unmask the effects of a possible inhibitor.

The data appearing in Figures 7a and 7b show a significant inhibition of background 5-HT release for the different PEC with and without PPD ($p < 0.02$, t-test), with a mean inhibition of $16\% \pm 2.5$. As was true in Figure 6, in Figure 7a the age of the PEC had no significant effect on the inhibition. The different supernatant dilutions did not uncover any agonist activity; the dilutions only had the expected effect of decreasing release at the higher dilutions. As was also seen in Figures 5 and 6, a comparison of Figures 7a and 7b shows similar inhibition independent of the presence of antigen. However, the mean inhibition of $16\% \pm 3$ in Figure 7 is significantly less than the mean inhibition of $44\% \pm 8$ in Figures 5 and 6 ($p < 0.01$, t-test). Thus the different incubation conditions in Figure 7 (higher cell concentration,

incubation in conical tubes) must account for the decreased amount of inhibition. It is unclear whether this represents a decreased titer of an inhibitor or an increased titer of a potential lymphokine which causes serotonin release from mast cells.

Experiments attempting to identify macrophages as an intermediary in the activation of mast cells by T-cells: An alternate hypothesis to mast cell activation via a T-cell derived lymphokine is that T-cells must interact first with macrophages, either via direct contact or a factor, and it is the macrophage which then activates mast cells. The PEC used in the previous experiments were employed as a source of macrophages.

In Table 3, various T-cell derived supernatants, obtained from Dr. Charles Janeway and Drs. Monte Meltzer and Edward J. Leonard, were incubated with mast cells. These supernatants are known to contain various kinds of lymphokines. Aliquots of these supernatants were also preincubated with CFA and IFA-induced PEC and then these supernatants were exposed to mast cells. Since the incubation conditions were similar to those in Figures 5 and 6, inhibition of background serotonin release from mast cells would be predicted with the media + PEC supernatant. The mean inhibition in Table 3 for media + PEC compared to media is 43%, which is virtually identical to the inhibition of 44% in Figures 5 and 6. Inhibition of background release was also present in eight of eight instances when the various T-cell supernatants were first preincubated with PEC. This inhibition of background release was, however, present in varying amounts for the different supernatants. There is no reproducible difference between the inhibition induced by IFA-PEC or CFA-PEC. As was the case in Figure 7 where there was less

inhibition than in Figures 5 and 6, it is unclear why some supernatants in Table 3 caused less inhibition of background release than other supernatants. Possible explanations for this observation include a lower titer or partial destruction of the inhibitory factor, a change in the recognition or uptake of the inhibitory factor by the mast cells, or the presence of some factor with an agonist activity.

In the experiments above, attempts were made to isolate various cell interactions in vitro in an effort to generate a factor causing mast cell secretion. Another protocol, which more closely reproduces the in vivo model of mast cell secretion in DTH, is to allow T-cells, antigen, macrophages and mast cells to interact directly. Spleen cells from animals receiving an optimal immunization for DTH elicitation (0.2ml of 0.01% SRBC) served as the source of T-cells. Normal spleen cells and spleen cells from animals receiving an immunization promoting antibody formation (0.2ml of 10% SRBC) were also used. CFA-SRBC-PEC which had been exposed to SRBC one hour prior to sacrificing the animal served as the antigen presenting macrophages. In Figure 8a, ^3H -5HT labelled mast cells were pelleted with the CFA-SRBC-PEC and spleen cells to facilitate cell-cell contact. The incubation period was 90 minutes. The results show serotonin release almost two times background release when either immune or normal spleen cells were incubated with mast cells, regardless of the presence of PEC. The incubation of PEC and mast cells caused serotonin release approximately equal to that of the media control.

Since mast cells constitute only 3-5% of the peritoneal cells, efforts were made to maximize the probability of a mast cell coming into contact with an antigen presenting macrophage. In Figure 8b,

CFA-SRBC-PEC and spleen cells were preincubated for 24 hours in round bottom tubes. The supernatant and non-adhered cells were then poured off, and the mast cells were spun down onto the adhered cells (presumably antigen presenting macrophages. The results show inhibition of background 5-HT release for all the different combinations of spleen cells and macrophages. In Figure 8c, a similar protocol to that in Figure 8a was followed, except that the mast cells, macrophages and spleen cells were incubated for 24 hours. With this long mast cell incubation period, the media background release, 28%, was considerably higher than in the 90 minute incubations. Interestingly, all the combinations of CFA-SRBC-PEC and immune or normal spleen cells, and PEC and the different types of spleen cells separately, showed strong inhibition of background release, with a mean inhibition of 54%. Based upon the three experiments in Figure 8, there is a progression from the modest agonist activity apparent after a 90 minute incubation to the strong inhibition of background 5-HT release apparent after 24 hours. In both Figures 8b and 8c, maximal inhibition occurs with normal spleen cells by themselves, perhaps indirect evidence, when considered with the small amount of enhanced serotonin release seen in Figure 8a, supporting the presence of an agonist in the immune cells.

Experiments attempting to identify an IgE-like material on mast cells:

Another hypothesis for T-cell - mast cell collaboration is that an IgE-like material attaches to mast cells and, upon antigenic challenge, causes mast cell degranulation. Mast cells were collected from mice which had received the optimal immunizing dose for DTH

elicitation, 0.01% SRBC. It was decided to use a 100 fold excess of SRBC ($5 \times 10^7/\text{ml} = 1\%$ SRBC) to assure erythrocyte contact with mast cells which constitute 3 - 5% of the peritoneal lavage.

Day four 0.01% SRBC mast cells and normal mast cells were incubated with 1% SRBC and 1% HRBC under different conditions as depicted in Table 4a. It is important to note that the presence of RBC in the pellet containing the ^3H -5HT labelled mast cells interfered with the ability of the B-counter to detect radioactivity by a factor of approximately two. This is not significant in the comparison of individual data in Table 4 because RBC were present in all the assay tubes. However, to compare data in Table 4 to experiments done without RBC, it would be necessary to divide the serotonin percent release in Table 4 by two. This would convert the 5-HT release for normal mast cells + 1% RBC to about 5.5%, a figure compatible with the value for background release in Locke's solution in other experiments. When incubated with SRBC, mast cells from mice immunized to optimally express DTH had a significantly greater serotonin release, under all the incubation conditions, than mast cells from normal mice ($p < 0.02$, t-test). Mast cells from mice immunized with SRBC also had a significantly greater 5-HT release when exposed to HRBC as compared to normal mast cells ($p < 0.02$, t-test). This cross-reactivity was also seen under the various incubation conditions.

Further studies were performed using different types of mast cells. Previous studies [7] have demonstrated good DTH reactions in mice four days after 0.01% SRBC immunization. DTH was, however, virtually undetectable 18 days after immunization. In addition,

mice four days after 10% SRBC immunization were shown to also have poor DTH reactions. Table 4b compares 0.01% SRBC immunized mice; mast cells were harvested four and 18 days after immunization. When incubated with SRBC, mast cells from mice 18 days after receiving optimal DTH elicitation doses (0.01% SRBC) released 8.2% serotonin compared to a mean of 16.6% for mast cells four days after immunization. Thus the increase in serotonin release four days after immunization is transient, an observation consistent with the SRBC model for DTH [7]. The 5-HT release after SRBC incubation with mast cells from mice 18 days after 0.01% SRBC immunization is not significantly different from that for normal mast cells, 11.5%. The serotonin release after SRBC incubation with mast cells from mice previously immunized with 10% SRBC (an antibody inducing dose) was 10.7%, also not significantly different from normal mast cells. A 0.01% SRBC immunization thus seems to induce a material which coats mast cells and, upon antigenic challenge, cause 5-HT release. This property is highly reminiscent of IgE. This IgE-like material is transiently present on the surface of mast cells, not present after classic antibody inducing immunizing doses, and it cross-reacts with HRBC.

Thus these in vitro experiments with the different kinds of mast cells appear to correspond to the in vivo SRBC model for DTH. There was an enhanced serotonin release after SRBC incubation with mast cells from mice four days after 0.01% SRBC immunization, a dose known to induce good DTH reactions. There was baseline serotonin release (equivalent to 1% SRBC + normal mast cells) from mast cells collected from mice 18 days after 0.01% SRBC immunization, or four days after 10% SRBC immunization, conditions known to result in poor DTH reactions.

Further experiments using anti-immunoglobulin antibodies incubated with the various types of immune mast cells were performed to detect any antibodies on the surfaces of mast cells. The data is presented in Table 5. There is essentially no difference between the different types of mast cells in their ability to be stimulated by 48/80. When rabbit anti-mouse IgE was incubated with the various mast cells, the day four 0.01% SRBC mast cells, interestingly, showed the highest serotonin release, 29%. The same observation is true for the goat anti-mouse IgG, but at a much lower level, 5.3%. It is possible that the goat anti-mouse IgG is not specific for IgG, but is reacting with light chain determinants in the IgE-like material. It is unclear whether the anti-mouse IgE and anti-mouse IgG were actually detecting antibody on the surfaces of day four 0.01% SRBC mast cells. The day 18 0.01% SRBC or the day four 10% SRBC mast cells might be expected to have more antibody on their surface because of, respectively, the longer period of time between immunization and testing, and the higher immunization dose. The anti-mouse IgE did cause increased release from the day 18 0.01% SRBC and the day four 10% SRBC mast cells as compared to normal mast cells. This enhanced release may represent antibody on the surfaces of these mast cells. The anti-mouse IgE is, however, clearly reacting more strongly to some immunoglobulin-like factor on the day four 0.01% SRBC mast cells that is not present on the other types of mast cells.

The results of Table 5 where the anti-mouse IgE and anti-mouse IgG caused the greatest ^3H -5HT release from the day four 0.01% SRBC mast cells are similar to the results of Table 4b

where the SRBC also reacted maximally with the day four 0.01% SRBC mast cells. Thus the anti-mouse antibody studies provide additional evidence for an IgE-like material present on the surfaces of day four 0.01% SRBC mast cells.

A further observation from the antibody studies was that the day 18 0.01% SRBC mast cells seemed to be sensitized to the combination of anti-DNP IgE + anti-mouse IgE. Anti-DNP-IgE, when incubated by itself with mast cells, caused no increase in 5-HT release above background. When preincubated with anti-DNP IgE and then incubated with anti-mouse IgE, the day four 0.01% SRBC mast cells, the day four 10% SRBC mast cells, and the normal mast cells released 3% - 6% more serotonin than they did without the anti-DNP IgE preincubation. However, when preincubated with anti-DNP IgE, the day 18 0.01% SRBC mast cells released 22% more serotonin after incubation with anti-mouse IgE. The etiology of this heightened sensitivity to antibody mediated serotonin release is uncertain. It may represent increased expression and/or synthesis of F_c receptors, or increased mast cell degranulation for a given amount of antibody - antigen interaction.

DISCUSSION

Based on previous experiments [22,55] a hypothesis was formulated that the local release of serotonin from tissue mast cells was required to elicit delayed-type hypersensitivity in mice. Evidence has been presented that sensitized T-lymphocytes interact with mast cells leading to mast cell degranulation and release of serotonin. Subsequent experiments [4] have shown that the serotonin caused separation of endothelial cells to allow the diapedesis of bone marrow derived effector cells. The present study was undertaken to further characterize the T-cell - mast cell interaction in DTH, and to obtain an in vitro correlate for this interaction.

Attempts to identify a lymphokine which causes serotonin release from mast cells

Based on the hypothesis that a T-cell derived lymphokine caused serotonin release from mast cells, initial experiments tried to identify such a lymphokine. In Tables 1 and 2, supernatants were generated from immune lymph node cells in culture with antigen and from T-cell lymphoma and hybridoma cell lines. These supernatants were previously established to contain various lymphokines. When incubated with ^3H -serotonin labelled mouse mast cells, these supernatants did not augment background (spontaneous) serotonin release.

Identification of an inhibitor of background serotonin release from mast cells

The next group of experiments utilized a heterogeneous population of cells; peritoneal exudate cells induced by complete

or incomplete Freund's adjuvant. PEC contain almost equal numbers of neutrophils, lymphocytes and macrophages (mononuclear cells with vacuolated cytoplasm, Figure 2). Unexpectedly, when supernatants from cultures of PEC + PPD were incubated with mast cells, there was significant inhibition of background release of ^3H -5HT ($p < 0.01$) (Figures 5 and 6). This inhibition was independent of the presence of antigen (PPD), independent of the type of PEC (whether induced by CFA or IFA), and was apparent after PEC culture periods of 24 or 48 hours. The mean inhibition of background release was 44%. Because of the heterogeneous cell population, it is unclear whether this inhibitory activity originated from the lymphocytes, neutrophils, or the macrophages.

A recent report [59] has identified macrophages as playing a significant regulatory role in immune functions through the secretion of both enhancing and inhibiting factors. The system studied involved murine peritoneal natural killer (NK) cell activity against mouse lymphoma cells. A few days after the intraperitoneal injection of BCG (*Mycobacterium bovis*, strain Bacille Calmette-Guerin), an augmentation of the peritoneal NK activity was observed. It was found that BCG-induced peritoneal macrophages secreted large quantities of prostaglandin E_2 (PGE_2). In vitro, PGE_2 inhibited BCG-induced peritoneal NK cell activity. Further experiments revealed that indomethacin and aspirin significantly potentiated the BCG-induced enhancement of NK activity by inhibiting the synthesis of PGE_2 . Thus the macrophage appears to be crucial in regulating NK activity in BCG infected mice via both enhancing and inhibitory factors.

In another system [25,26], somewhat more analogous to the secretion of vasoactive amines, an inhibitor of histamine release was isolated from eosinophils. In this system, eosinophil-rich fractions of human leukocytes were incubated with specific allergen or anti-IgE. The cell-free supernatants were then incubated with human leukocytes assaying for the inhibition of anti-IgE induced histamine release. This inhibition was found to be related to prostaglandin because indomethacin blocked the immune release of the inhibitor from eosinophils. Thus it appears that eosinophils, as well as macrophages, can assume a regulatory role in the immune system.

PGE₁ and PGE₂ have been shown to be important regulators of immune responses by modifying antibody production, the mitogenic stimulation of lymphocytes, and delayed hypersensitivity reactions [23]. Mouse peritoneal macrophages have been demonstrated to synthesize prostaglandin E [31]. It is thus possible that the factor inhibiting the background release of serotonin from mast cells may be related to a prostaglandin originating from peritoneal macrophages. An interesting follow-up experiment would be to incubate the PEC in the presence of a prostaglandin synthesis inhibitor (a cyclooxygenase inhibitor), such as indomethacin or aspirin, and then incubate the supernatant with ³H-5HT labelled mast cells. It is possible that by abolishing the inhibitory activity, a material with agonist activity may become evident.

The experiments depicted in Figure 7 are similar to those in Figures 5 and 6 except that the PEC were incubated at higher cell concentrations and in conical tubes instead of flat-bottomed

wells. Certain experiments have demonstrated the critical need for achieving intimate cell-cell contact. In vivo reactions, such as DTH, occur in stable and highly organized local environments, e.g. the mammalian epidermis. However, in in vitro cell cultures, achieving the same potential for intimate physical interaction that is provided by the inherent nature of epidermis, is no simple task. The critical nature of cellular interactions is manifest in experiments assaying for the production by sensitized T-cells of macrophage migration inhibitory factor (MIF). By increasing cellular density, and by permitting spontaneous sedimentation in a conical test tube, the MIF activity of a supernatant from cultures of lymphocytes and antigen was augmented [39,47,48] by a factor as great as 10^{10} [47]. The production of MIF was dependent on the presence of antigen (PPD) in the incubation with the sensitized cells; MIF activity was absent when antigen was incubated with non-sensitized cells. The amplification effect was abolished by dispersing the cell suspension over the large area of a flat-bottomed flask or by shaking the cell suspension. Thus the amplified migration inhibition effect was negated by preventing prolonged intimate cell-cell interactions.

Using conical tubes and higher cell concentration for the incubation of PEC + PPD, Figure 7 shows significant inhibition of background release of serotonin ($p < 0.02$) with a mean inhibition of 16%. Interestingly, this inhibition was significantly less than the inhibition observed in Figures 5 and 6 ($p < 0.01$), where the PEC were incubated at lower cell concentrations in flat-bottomed wells. It remains unclear whether the decrease amount of inhibition

in Figure 7 represents a decreased titer of an inhibitor, or an increased titer of a substance which causes serotonin release from mast cells. The addition of a prostaglandin synthesis inhibitor to the incubation of PEC in conical tubes at high cell concentrations may be able to uncover an agonist activity.

Table 3 presents experiments in which various T-cell derived supernatants, known to contain different lymphokines, were incubated either directly with mast cells, or first with PEC and then with ^3H -5HT labelled mast cells. The results are similar to Figure 7. In Table 3, there are different degrees of inhibition of background serotonin release when the various T-cell supernatants were preincubated with PEC. As in Figure 7, this observation may represent a decreased titer of an inhibitor or an increased titer of a factor with agonist activity. The addition of a prostaglandin inhibitor to the PEC cultures would again be of interest.

The experiments in Figure 8 attempt to reproduce the SRBC model of DTH by interacting mast cells, sensitized T-cells (spleen cells four days after an optimal DTH elicitation SRBC dose), antigen-presenting macrophages (CFA-SRBC-PEC) and free antigen (SRBC). Proper cell-cell interaction is a major potential problem with so many different cell types. In Figure 8a, the ^3H -5HT labelled mast cells, spleen cells and PEC were pelleted to facilitate cell-cell contact and incubated for 90 minutes. In Figure 8c, the three types of cells were incubated for 24 hours. Figure 8a demonstrates modest enhancement of 5-HT release above background levels with either immune or normal spleen cells, though it is not statistically significant. Figure 8c, on the other hand, shows marked inhibition

of background 5-HT release with all the various combinations of PEC and spleen cells.

This progression from modest enhancement of serotonin release apparent after a 90 minute incubation period to the significant inhibition seen after the 24 hour incubation period suggests the possibility that an inhibitory factor was produced over time. Different incubation periods between 90 minutes and 24 hours would be helpful in defining the time course of production of the potential inhibitor. As was discussed earlier, macrophages are known to produce factors that can enhance immune responses and other factors that can inhibit them. It is possible that an agonist for 5-HT release may be produced early during the incubation period and the inhibitory activity becomes evident only later, masking the agonist activity. Prostaglandin synthesis inhibitors would also be valuable in these experiments by possibly unmasking an agonist activity.

Identification of an IgE-like substance on the surface of mast cells

The previous experiments are based on the hypothesis that mast cell activation occurs via a T-cell derived lymphokine or via an initial T-cell - macrophage interaction, with subsequent mast cell activation. Another hypothesis for T-cell - mast cell collaboration is that it occurs via IgE or an IgE-like material. There is a substantial amount of evidence that the mast cell activation and serotonin release leading to endothelial gap formation in the SRBC model of DTH is T-cell dependent, and not mediated by B-cell products such as IgE. In the actively sensitized animal challenged at day four to express DTH, there is little

evidence for B-cell activation as determined by antibodies to SRBC [6] or by induction of antigen binding rosette forming B-cells [14]. In addition, mice treated with anti-IgM antisera and rendered unable to produce antibodies are able to express DTH [12,35].

There is now evidence that T-cell membranes contain immunoglobulin-like molecules [43], which possess antigen binding properties [20]. If this T-cell "receptor" or a similar substance were able to be released from cells and attach to mast cells via an IgE-like activity, it may be able to trigger mast cell activation by binding antigen. This reasoning about T-cell derived materials is speculative, but it should be considered, in light of the evidence against the participation of B-cells in the SRBC model of DTH, and in light of the results in Tables 4 and 5.

The experiments performed in Tables 4 and 5 reveal the presence of an IgE-like substance on mast cells collected four days after mice were immunized with 0.01% SRBC, an optimal dose for DTH elicitation. In Table 4a, after incubation with a 1% suspension of sheep erythrocytes, the ^3H -5HT release from these day four 0.01% SRBC mast cells was significantly greater than the ^3H -5HT release from normal mast cells ($p < 0.02$). However, the ^3H -5HT release, after incubation with SRBC, from day 18 0.01% SRBC mast cells or from day four 10% SRBC mast cells was not different from the ^3H -5HT release from normal mast cells (Table 4b). As previously mentioned, the transient presence of the substance coating mast cells from mice immunized with 0.01% SRBC and the baseline response (equivalent to ^3H -5HT release from normal mast cells incubated with 1% SRBC) of the day four 10% SRBC mast cells correspond remarkably closely to

the in vivo SRBC model of DTH [7]. In this model, optimal DTH responses are elicited from mice four days after 0.01% SRBC immunization, while poor DTH responses occur 18 days after 0.01% SRBC immunization or four days after 10% SRBC immunization.

Thus it appears that there is an IgE-like factor on the surface of day four 0.01% SRBC mast cells which, upon exposure to antigen, SRBC, triggers 5-HT release. It is unlikely that this factor is an antibody; the other kinds of mast cells (day four 10% SRBC and day 18 0.01% SRBC) from immunized animals would be expected to produce more antibody because of the higher immunization dose or the longer period of time between immunization and testing. The results with the day four 10% SRBC and the day 18 0.01% SRBC mast cells need to be repeated to confirm that the 5-HT release from these mast cells incubated with 1% SRBC is not significantly different from the 5-HT release from normal mast cells.

Evidence supporting the hypothesis based on Table 4 that the IgE-like factor is distinct from conventional antibodies comes from the experiments in Table 5. The results in Table 5 show that the rabbit anti-mouse IgE and the goat anti-mouse IgG caused the greatest ^3H -5HT release when incubated with the day four 0.01% SRBC mast cells. These results are similar to the results of Table 4b where the SRBC also reacted maximally with the day four 0.01% SRBC mast cells. It is possible that the SRBC effect and the anti-immunoglobulin antibody effect may not be due to the same substance on the surface of the day four 0.01% SRBC mast cell. There was a

considerably lower level of ^3H -5HT release from mast cells incubated with the goat anti-mouse IgG compared to the rabbit anti-mouse IgE. The goat anti-mouse IgG may not be specific for IgG and could be, to a limited extent, cross-reacting with light chain determinants in the IgE-like material. Decreased reagent purity and lower specific activity of the goat anti-mouse IgG preparation could also account for the low level of 5-HT release with the goat anti-mouse IgG.

The rabbit anti-mouse IgE did react maximally with the day four 0.01% SRBC mast cells, but it also caused substantial ^3H -5HT release from the day four 10% SRBC and day 18 0.01% SRBC mast cells. The 5-HT release from the anti-mouse IgE incubation with the day four 10% SRBC or day 18 0.01% SRBC mast cells may result from the presence of conventional antibody on the surface of these mast cells. It is quite unlikely that more antibody would be present on the surface of the day four 0.01% SRBC mast cells because of the smaller immunization dose and the shorter time interval between immunization and testing compared to the other immune mast cells. Thus, as was previously seen with the incubations with 1% SRBC, it appears that an IgE-like material which reacts with antigen and with anti-mouse immunoglobulin, most likely not conventional antibody, is coating the surface of day four 0.01% SRBC mast cells. The experiments with the anti-immunoglobulin antibodies need to be repeated to confirm a significant difference for the four types of mast cells. The precise specificity of the anti-immunoglobulin antibodies also needs confirmation.

Further evidence against this IgE-like factor being a conventional antibody comes from the mast cells incubations with 1% suspensions of horse erythrocytes. The day four 0.01% SRBC mast cells had a significantly greater ^3H -5HT release than normal mast cells, not only when incubated with SRBC ($p < 0.02$), but also when incubated with HRBC ($p < 0.02$) (Tables 4a and 4b). After incubation with HRBC, the ^3H -5HT release from day four 10% SRBC and day 18 0.01% SRBC mast cells was not significantly different from the 5-HT release from normal mast cells (Table 4b). Thus the day four 0.01% SRBC mast cells exhibit crossreactivity for HRBC

In the SRBC model of murine DTH, there is some evidence for crossreactivity to HRBC. Mice immunized with 0.01% SRBC failed to express significant reactions whe footpad tested with HRBC in the usual challenge dose of a 20% erythrocyte suspension [7]. However, testing with 50% HRBC in mice immunized with SRBC did produce small but significant cross-reactive DTH responses [7]. Thus DTH reactions were specific but higher challenging doses could elicit cross-reactive DTH responses. Instead of incubating the day four 0.01% SRBC mast cells with 1% erythrocyte suspensions, it might be informative to use other erythrocyte dilutions, such as 0.1% and 0.01%, to determine whether the response to the homologous antigen would remain intact. Under these conditions, the cross-reactivity to HRBC might be eliminated. Mice could also be immunized with 0.01% HRBC to determine whether these mast cells would exhibit crossreactivity for SRBC.

Delayed hypersensitivity responses to various proteins often show cross-reactions between antigens that do not cross-react with the specific antibody [21,46 ,51]. In one system,

DTH responses were measured after guinea pig immunization to horse cytochrome c protein. The animals were subsequently skin tested with cytochrome c from horse and other species. It was found that significant cross-reactions existed when there were only minor amino acid sequence differences between the horse cytochrome c and the cytochrome c of the species skin tested [51]. There was less cross-reactivity when there were more differences between the aminoacid sequences. It is interesting that a high degree of specificity has been shown in DTH reactions involving haptenated proteins such as dinitrophenyloligolysine [54]. The flagellin proteins from two different species of Salmonella, although observed to produce very weakly cross-reacting antibodies, had strongly cross-reactive DTH responses [46]. Similar cross-reactivity has been reported to occur between serum albumins [21]. In a system studying a different T-cell function, there was extensive cross-reactivity in the cytotoxic T-cell response to a variety of serologically distinct influenza A viruses [19]. Thus there is data to suggest cross-reactivity of T-cells in delayed hypersensitivity while it is generally accepted that antibodies only react with the one specific antigen.

The cross-reactivity of the IgE-like factor coating the day four 0.01% SRBC mast cells thus provides additional, although by no means firm, evidence against the factor being a conventional antibody. The IgE-like factor is reacting with some antigenic determinant or component of the horse and sheep erythrocyte, an interaction that results in mast cell activation and serotonin release. Erythrocytes from other species, such as goats and

humans should also be incubated with the day four 0.01% SRBC mast cells to determine the extent of the cross-reactivity.

The IgE-like factor thus has properties that are not characteristic of antibodies: cross-reactivity, lack of appearance after antibody inducing immunization doses, and transient presence after DTH eliciting doses. These properties are actually more analogous to a hypothetical T-cell derived mediator of DTH. These properties, when combined with the evidence against the participation of B-cells in DTH, suggest that this factor could be a T-cell derived product which possesses the ability to interact with antigen.

Summary

The present study has attempted to identify in vitro T-cell interaction with mast cells that results in the release of serotonin. Experiments with peritoneal exudate cells revealed the presence of an inhibitor of background (spontaneous) serotonin release from mast cells along with some indirect evidence for the presence of a substance that could actually induce serotonin release or inhibitor inactivation. Many possible approaches for further investigation have already been discussed. Further characterization of the inhibitory activity might involve incubation of the PEC supernatants with compound 48/80 or with antibody and antigen to determine whether the inhibitory factor was also active against the ability of well characterized agonists to mediate serotonin release. Other experiments performed identified an IgE-like substance on the surface of mast cells from mice immunized to optimally express DTH. This substance interacted

with a multivalent antigen and with anti-mouse IgE and anti-mouse IgG. Arguments were presented that this substance could be derived from T-cells. However, it is conceivable that this substance is IgE. It may be preferentially induced by immunization with low antigen doses, and it may possess a short half life.

Further study of this IgE-like substance might include identifying its presence in mice rendered agammaglobulinemic by treatment with anti-IgM antisera. Other types of antigen, such as protein or other particulate antigens, could be used to immunize mice and test whether the cross-reactivity is simply a function of the geometry or inherent similarities between SRBC and HRBC. Additionally, different kinds of anti-immunoglobulin antibodies, such as anti-idiotypic antibodies, could be used to further elucidate the nature of the IgE-like material. Recently, antibodies have also been produced against antigen specific T-cell derived molecules [43]. Such antibodies could also be incubated with day four 0.01% SRBC mast cells.

It has recently been revealed that mast cells, when treated with amitriptyline and other psychotropic agents, can secrete serotonin in the absence of histamine release and in the absence of overt degranulation [8]. Tritiated serotonin release was measured exclusively in the present study. Mast cells' capacity for this differential release has not yet been identified in any physiologic system. It would be of interest to assay for concomitant histamine release from the incubation of day four 0.01% SRBC mast cells and antigen or anti-immunoglobulin antibody.

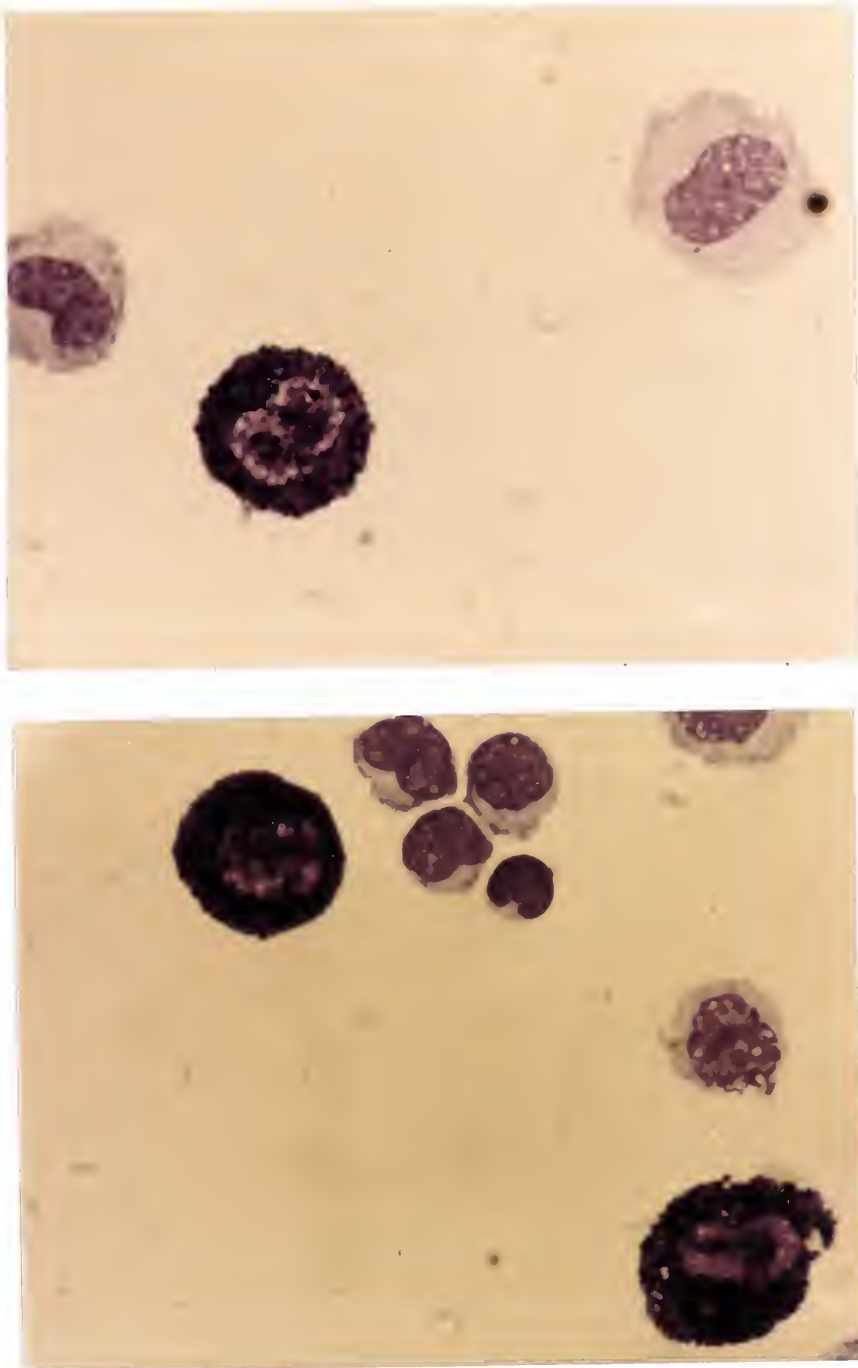


FIGURE 1: Photographs of mouse mast cells stained with Wright-Giemsa. Note the large number of purple-staining granules in the cytoplasm of the mast cell. Other cells are lymphocytes and other mononuclear cells. Magnification x 400.

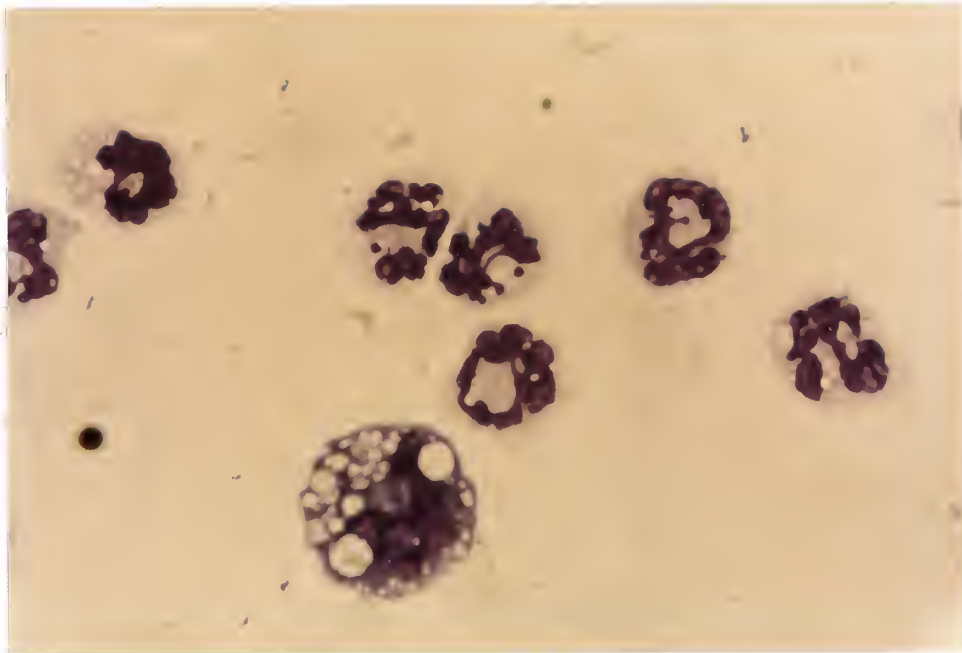
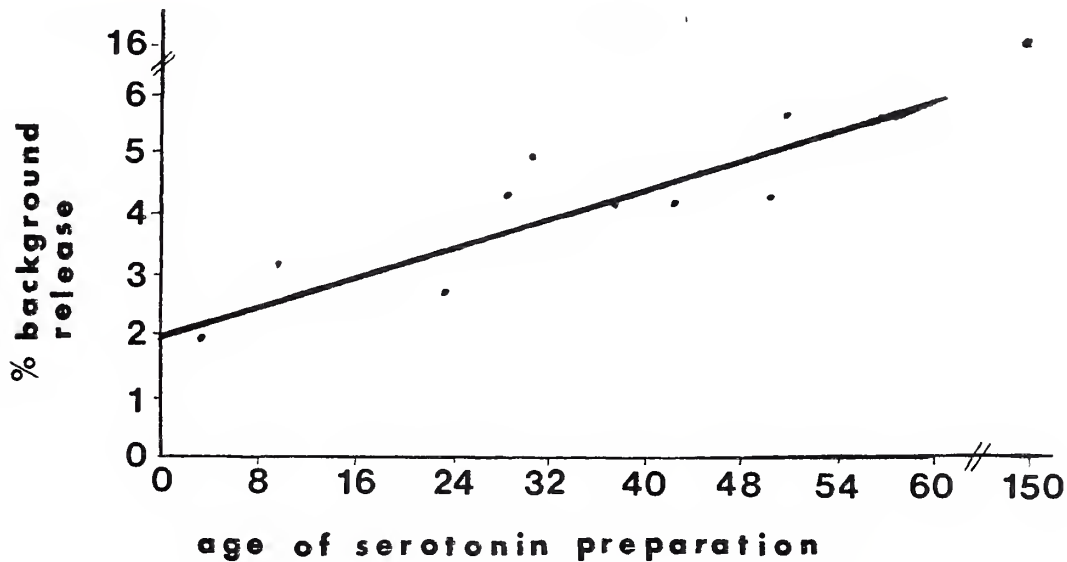


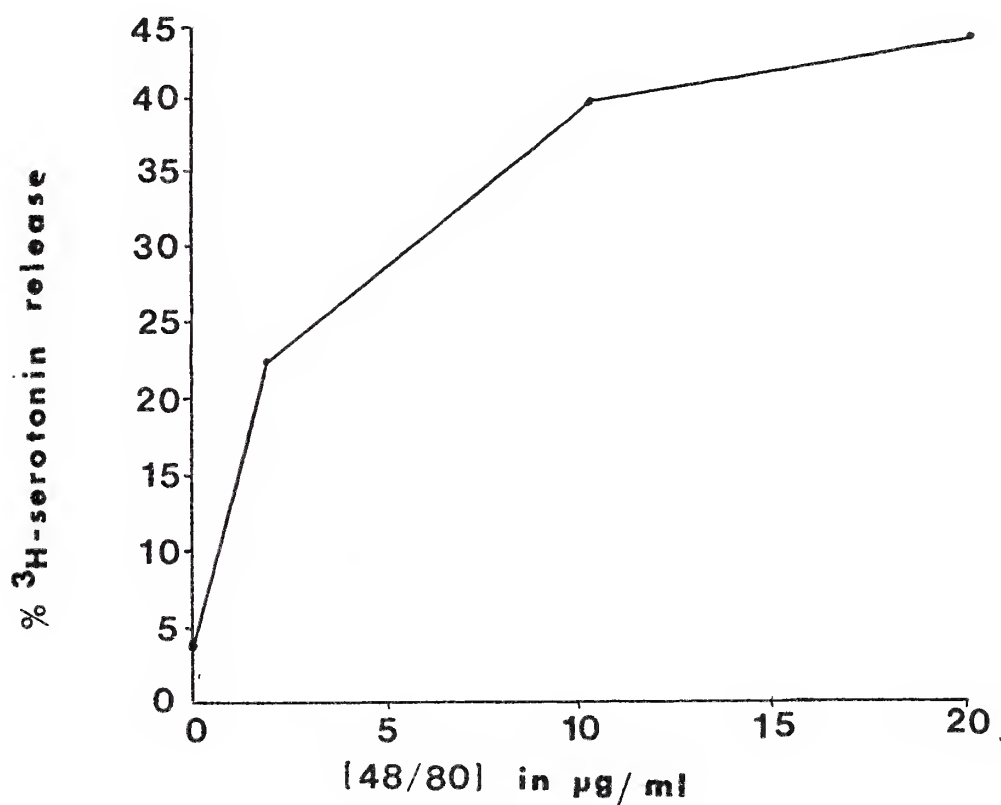
FIGURE 2: Photograph of mouse peritoneal exudate cells, induced by complete Freund's adjuvant. Note large mononuclear cell with vacuolated cytoplasm. Other cells are neutrophils. Magnification x 400.

FIGURE 3: Background (spontaneous) release of ^3H -serotonin in Locke's solution as a function of the age of the serotonin preparation*



* ^3H -serotonin labelled mast cells were incubated in Locke's solution for 90 minutes at 37°C . The age of the serotonin preparation represents the number of days after delivery from New England Nuclear. The line drawn represents the linear regression line.

FIGURE 4: ^3H -serotonin release from mast cells as a function of the concentration of compound 48/80*



* ^3H -serotonin labelled mast cells were incubated with compound 48/80 at the indicated concentrations for 90 minutes at 37°C .

Table 1: ^3H -serotonin release from mast cells incubated with ovalbumin induced supernatants*

<u>Type of supernatant</u>	<u>Supernatant dilutions</u>		
	<u>1:2</u>	<u>1:20</u>	<u>1:200</u>
OVA + LNC in a plate	6.6%	4.0%	4.0%
OVA + LNC in conical tubes	6.1%	4.2%	4.0%
Media	5.1%	4.1%	4.0%

*Supernatants were generated from a 24 hour incubation, in a plate or conical tubes, of 250ug/ml ovalbumin (OVA) with 2×10^7 inguinal lymph node cells (LNC)/ml. The lymph node cells were obtained from C57 female mice, 8 days after subcutaneous immunization in the tail of 50ug OVA + complete Freund's adjuvant. Aliquots of the supernatants and media were then diluted in Locke's solution and incubated with ^3H -5HT labelled mast cells for 90 minutes at 37°C .

Table 2: ^3H -serotonin release from mast cells incubated with T-cell lymphoma and hybridoma supernatants*

<u>Type of supernatant</u>	<u>Supernatant dilutions</u>	
	<u>1:2</u>	<u>1:100</u>
BW 5147 with serum	6.9%	4.9%
BW 5147 without serum	7.5%	4.7%
64C11 with serum	5.7%	4.7%
64C11 without serum	6.9%	5.3%
Media	7.3%	5.1%

*Supernatants were generated from T-cell lymphoma (BW5147) and hybridoma (64C11) cell lines supplied by Dr. Nancy Ruddie. These cells were incubated without antigen for 48 hours. Aliquots of the supernatants and media were then diluted in Locke's solution and incubated with ^3H -5HT labelled mast cells for 90 minutes at 37°C .

FIGURE 5: ^3H -serotonin release from mast cells incubated with supernatants from CFA-PEC or IFA-PEC + PPD*

Figure 5a

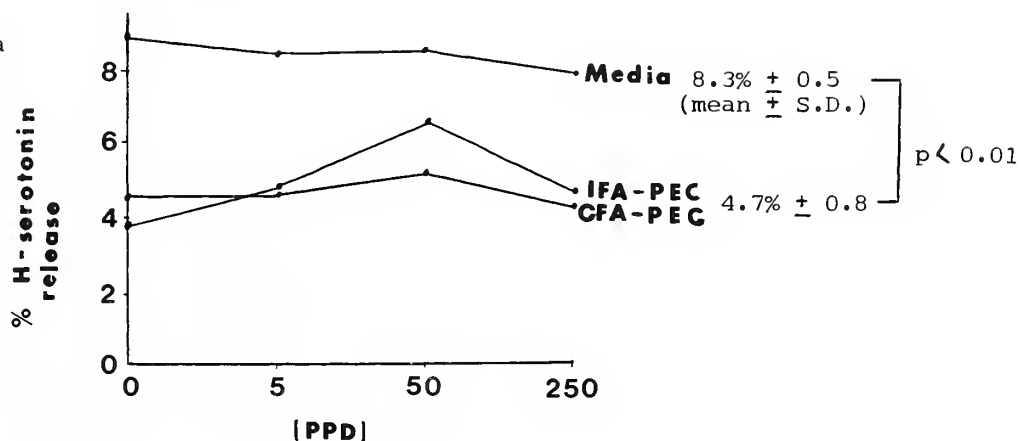
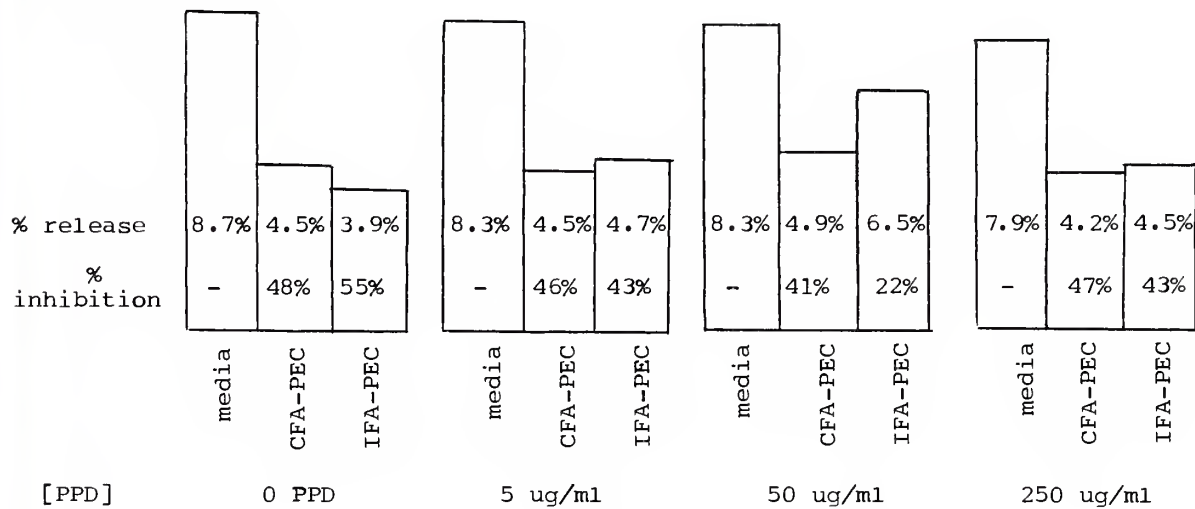


Figure 5b



*3 day CFA-PEC or IFA-PEC + PPD were incubated 24 hours at 37°C . in 3 cm wells. Then a 0.5ml aliquot of the supernatant was incubated with ^3H -5HT labelled mast cells for 90 minutes at 37°C .

FIGURE 6: ^3H -serotonin release from mast cells incubated with supernatants from 5 day or 9 day CFA-PEC + PPD*

Figure 6a

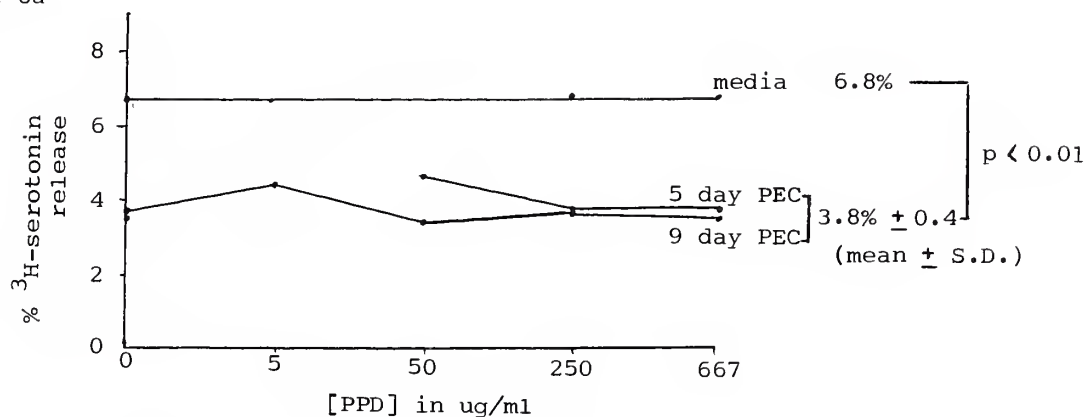
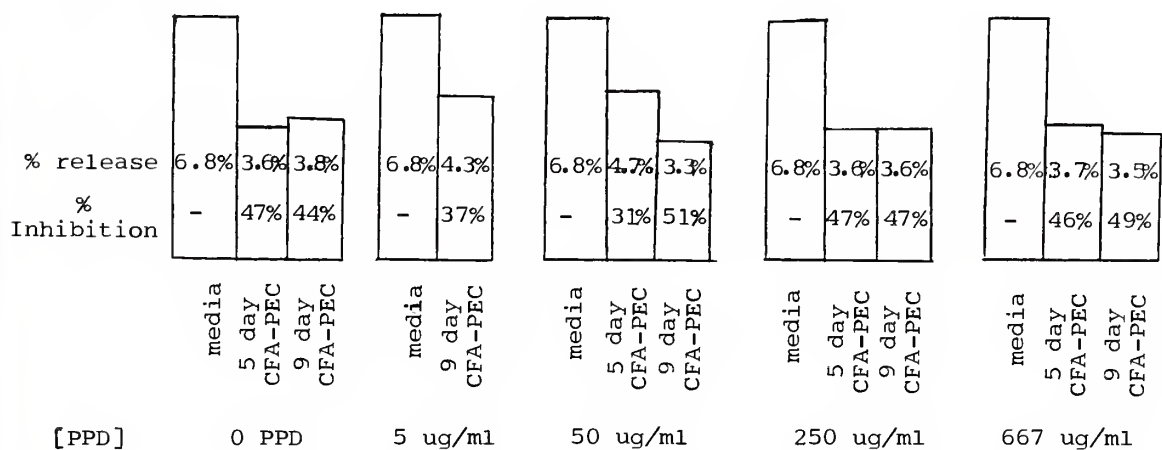


Figure 6b



*5 day or 9 day CFA-PEC + PPD were incubated 48 hours at 37°C . in wells. Then a 0.5ml aliquot of the supernatant was incubated with ^3H -serotonin labelled mast cells for 90 minutes at 37°C .

FIGURE 7: ^3H -serotonin release from mast cells incubated with supernatants from 5 day, 11 day, and 24 day CFA-PEC + PPD*

Figure 7a

Supernatant dilution

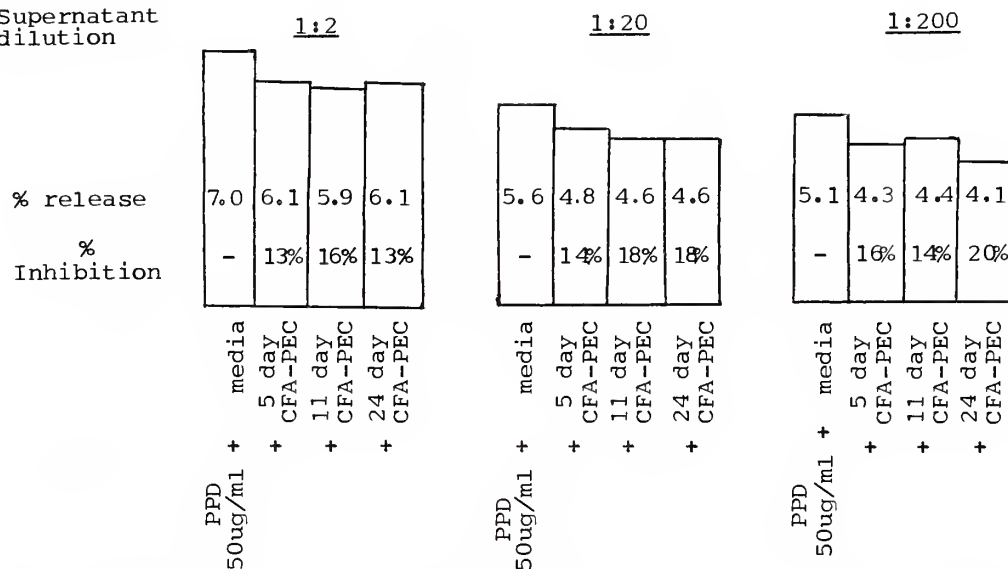
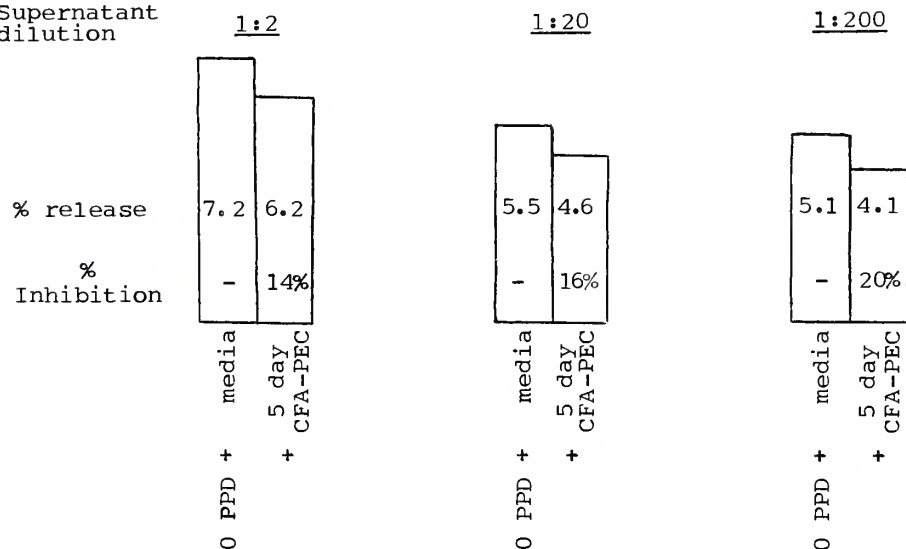


Figure 7b

Supernatant dilution



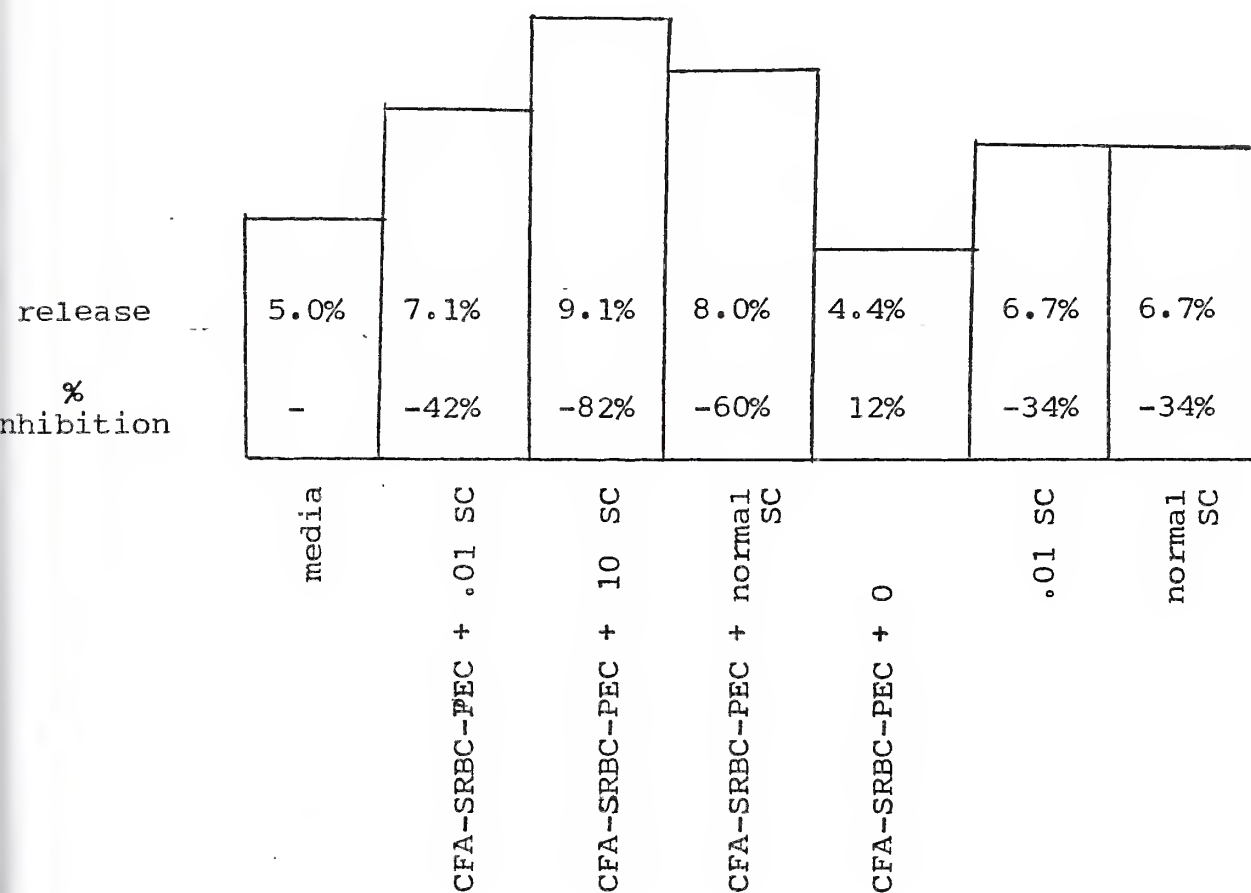
*5 day, 11 day, 24 day CFA-PEC + 50 ug/ml PPD were incubated 48 hours @ 37°C. in conical tubes, $15-20 \times 10^6$ cells/ml. Then ^3H -5HT labelled mast cells and different supernatant dilutions, 1:2, 1:20, 1:200, were incubated 90 minutes at 37°C.

Table 3: ^3H -serotonin release from mast cells incubated with T-cell derived supernatants and with supernatants generated from the incubation of these T-cell supernatants with peritoneal exudate cells*

Supernatant incubated with mast cells	T-cell derived supernatant								Media
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>	
T-cell supernatant	7.6%	5.5%	7.6%	4.5%	5.5%	5.0%	5.4%	5.4%	7.4%
Supernatant + CFA-PEC	3.3%	4.0%	4.3%	3.5%	4.7%	4.1%	4.8%	4.8%	4.5%
Supernatant + IFA-PEC	4.5%	4.1%	-	4.2%	4.0%	-	-	-	3.9%
Mean inhibition for supernatant + PEC	49%	26%	43%	15%	21%	18%	11%	11%	43%

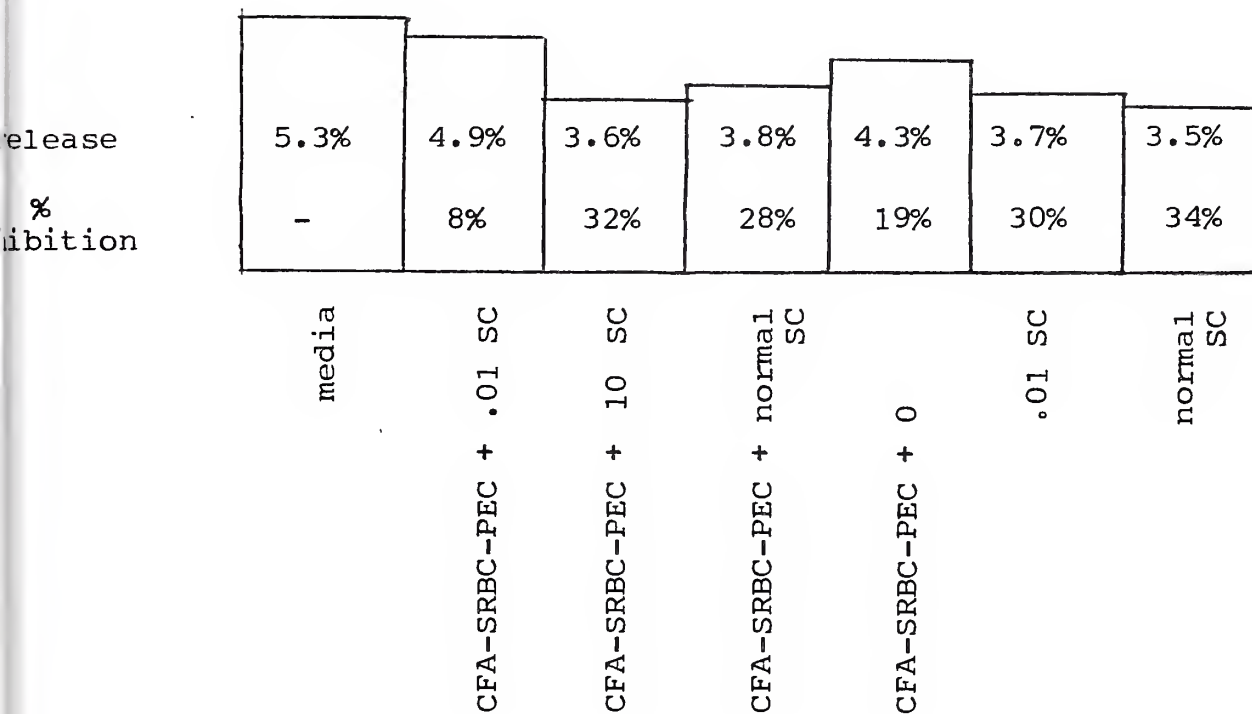
*T-cell derived supernatants #1 - #5 were obtained from Dr. Charles Janeway; supernatants #6 - #8 were obtained from Drs. Monte Meltzer and Edward J. Leonard. The original T-cell supernatants and media control were incubated with ^3H -5HT labelled mast cells for 90 minutes at 37°C . T-cell supernatants were also incubated with 3 day CFA-PEC and IFA-PEC, 7×10^6 cells/ml in wells for 24 hours at 37°C . Then aliquots of the supernatant - PEC were incubated with ^3H -5HT labelled mast cells for 90 minutes at 37°C .

FIGURE 8a: ^3H -serotonin release after incubation of mast cells, macrophages, spleen cells and antigen*



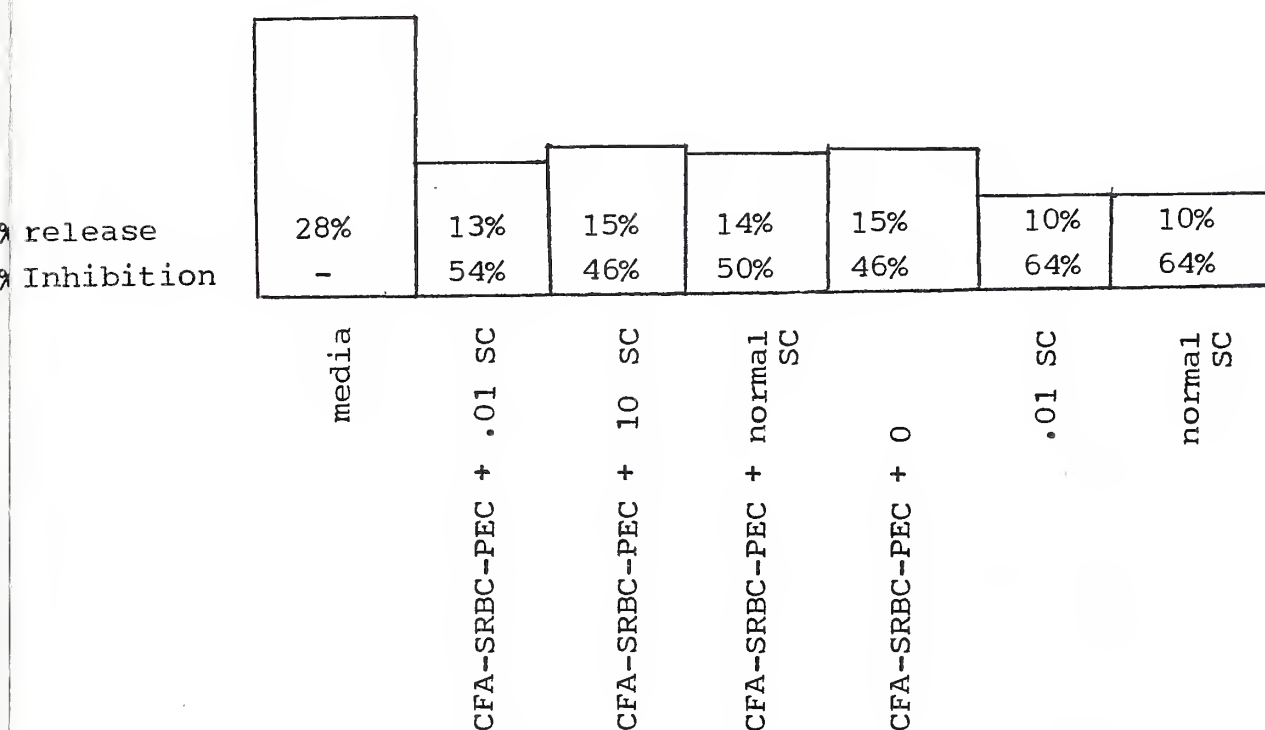
*5 day CFA-SRBC-PEC - $7 \times 10^6/\text{ml}$ and spleen cells - $7 \times 10^6/\text{ml}$ (4 days after 0.01% SRBC or 4 days after 10% SRBC immunization or after saline injection) and ^3H -5HT labelled mast cells - $1 \times 10^5/\text{ml}$ were pelleted in round-bottomed test tubes and incubated 90 minutes at 37°C .

FIGURE 8b: ^3H -serotonin release after incubation of mast cells, macrophages, spleen cells and antigen*



* 5 day CFA-SRBC-PEC - $7 \times 10^6/\text{ml}$ and 4 day .01, 10, normal spleen cells - $7 \times 10^6/\text{ml}$ were incubated in tubes for 24 hours at 37°C . Then the supernatant and non-adhered cells were poured off. ^3H -5HT labelled mast cells were spun down onto the adhered cells and incubated 90 minutes at 37°C .

FIGURE 8c: ^3H -serotonin release after incubation of mast cells, macrophages, spleen cells and antigen*



*The different combinations of PEC (macrophages), spleen cells (T-lymphocytes) and mast cells were incubated as in Figure 8a except that all the types of cells were incubated for 24 hours at 37°C.

TABLE 4: ^3H -5HT release after interaction between sheep or horse erythrocytes and mast cells from normal or immunized mice*

Antigen + mast cells	Incubation conditions for mast cells + RBC		
	Pelleted; 45min @ 4°C, 45min @ 37°C	Settling; 45min @ 4°C, 45min @ 37°C	Settling; 90min @ 37°C
SRBC + day 4 .01% SRBC mast cells	15.1% \pm 1.1	28.3% \pm 7.0	16.6% \pm 4.1
SRBC + normal mast cells	11.3% \pm 2.7	14.6% \pm 5.7	11.5% \pm 1.1
HRBC + day 4 .01% SRBC mast cells	13.8%	14.7%	17.3% \pm 2.6
HRBC + normal mast cells	10.5%	11.2%	12.6% \pm 1.6

Table 4b:

Mast cells	Antigen	
	SRBC	HRBC
day 4 .01% SRBC mast cells	16.6% \pm 4.1 (n=8)	17.3% \pm 2.6 (n=6)
normal mast cells	11.5% \pm 1.1 (n=6)	12.6% \pm 1.6 (n=4)
day 18 .01% SRBC mast cells	8.2%	9.6%
day 4 10% SRBC mast cells	10.7%	12.2%

*1% erythrocyte suspensions were incubated with various kinds of mast cells. Mast cells were collected 4 or 18 days after mice were immunized with .01% SRBC. Mast cells were also collected 4 days after mice were immunized with 10% SRBC. Mast cells from normal mice were also used. Incubation conditions were as described in Table 4a. In Table 4b, all erythrocytes + mast cells were incubated settling in tubes, 90 minutes at 37°C. Values are mean ^3H -5HT release \pm standard deviation. Number of experiments, n, comprising each mean is indicated in parenthesis.

TABLE 5: ^3H -serotonin release from mast cells from immunized animals after treatment with anti-immunoglobulin antibody*

<u>Mast cells</u>	<u>SRBC</u>	<u>HRBC</u>	<u>rabbit anti-mouse IgE</u>	<u>anti-DNP IgE + rabbit anti-mouse IgE</u>	<u>goat anti-mouse IgG</u>	<u>48/80</u>
day 4 .01% SRBC mast cells	16.6%	17.3%	28.6%	32.1%	5.3%	26.0%
day 18 .01% SRBC mast cells	8.2%	9.6%	8.1%	30.3%	2.7%	31.9%
day 4 10% SRBC mast cells	10.7%	12.2%	18.3%	23.5%	1.5%	29.3%
normal mast cells	11.5%	12.6%	4.2%	10.2%	2.9%	28.8%

*The different mast cells were incubated with erythrocytes for 90 minutes at 37°C ., as described in Table 4. The mast cells were also incubated with 48/80, 20 $\mu\text{g}/\text{ml}$, for 90 minutes at 37°C . The mast cells were incubated in Locke's solution for 45 minutes and then with rabbit anti-mouse IgE or with goat anti-mouse IgG for 45 minutes. The mast cells were also first incubated with anti-DNP IgE for 45 minutes. The assay tubes were then spun, the supernatant was poured off and the pellet was brought up in Locke's solution and incubated with anti-mouse IgE for an additional 45 minutes.

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